Biosorption of metals by activated carbon

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Contents

Introduction

Pollution by toxic heavy metals has been highlighted in recent years as an important environmental concern. The polluting activities of industry, responsible for the introduction of much of the toxic metal waste into water courses, have long gone relatively unchecked. Due to limited regulation in the past, waste strategies have relied upon dilution of the toxic components before release into natural water bodies with little effort to minimize the emissions of toxic metals. The intensification of industrial activity has meant that an increasing mass of waste is being released. In time even seemingly innocuous discharges are concentrated within the local aquatic flora and fauna reaching unacceptable levels. Awareness of the persistence in the environment and the deleterious health effects of these potentially toxic metals has heightened as progress has been made in understanding the complex inter-relations of metals, living organisms and the environment. The conventional views of pollution control in the U.K. are now being challenged as the Environmental Protection Act 1990, (Department of the Environment, 1991) sets out a broad range of legislation to

encourage industry toward waste abatement. Significant effort, including this project, is now being concentrated on the most suitable means of achieving the remediation of existing heavy metal pollution problems.

1.1 Metals in the environment

Few metals, notably potassium, magnesium, sodium and calcium, are required as macrominerals for the maintenance of osmotic balance and some structural integrity in all cells (Mertz, 1981; Hughes & Poole, 1989 a). Other, trace metals, although essential at very low levels for specific physiological roles in living organisms (Gadd, 1988; Hughes & Poole, 1989 b), may result in toxicity symptoms when overabundant or when substituted with non-essential metals such as the heavy metals (Sterritt & Lester, 1980; Volesky, 1990 a). The group of 'heavy metals' is an arbitrary classification and a misnomer, since it includes trace metals which are neither heavy in terms of atomic weight nor density, nor entirely metallic in character (e.g. arsenic). Generally the group includes metals other than those in groups I and II of the Periodic Table. Despite their relatively low abundance in the earth's crust the metals exert a disproportionally high influence and hence require careful monitoring and control (Volesky, 1990 a).

1.1.1 Toxic heavy metals

Heavy metals of particular concern, due to their prevalence in industrial wastes and increasing presence in natural waters, include cadmium, mercury, lead, chromium, copper, zinc and nickel (Kirk & Lester, 1984). The toxic health effects of what were considered the most dangerous of these (cadmium, mercury and lead) have already been widely reported. More recently the effects of chromium have been studied as its full polluting capacity has become apparent.

1.1.1.1 Cadmium

Cadmium, although accumulated by vegetation, has no known biological function (Vallee & Ulmer, 1972; Macaskie & Dean, 1982). It migrates easily into the environment and is strongly adsorbed by living organisms, making it amongst the

most toxic of metals (Volesky, 1990 a). Cadmium build-up in the organs is dangerous because of its long half-life which may be between 10 and 30 years (Moore & Ramamoorthy, 1984 a). Cadmium accumulation in the human body can produce chronic poisoning causing proteinuria (excretion of low molecular weight proteins) and formation of kidney stones. Exposure to high levels of cadmium has been linked to cancer (Piscator, 1981; Friberg & Kjellstrom, 1981). Sources of cadmium arise from manufacture of paint pigments, plastics, silver cadmium batteries and from the electroplating industry (Volesky *et al.*, 1993).

1.1.1.2 Chromium

Chromium is a toxic metal widely used in electroplating, tanning and as a biocide in the cooling water of power plants (Volesky, 1990 b; Nourbakhsh *et al.*, 1994). Electroplating and metal finishing wastes primarily contain hexavalent chromium (Huang & Bowers, 1978). As a pure metal it has no adverse health effects and the trivalent ion is an essential trace element for mammals for the potentiation of insulin (Mertz, 1981). Hexavalent chromium compounds cross cell membranes and are taken up easily by the lungs, digestive tract and skin (Sterritt & Lester, 1980; Baruthio, 1992). The reduction of Cr^{6+} to Cr^{3+} occurs within the cells (Debetto & Luciani, 1988) and this causes toxic effects including skin reactions, bronchial problems, lung cancer, gastroenteritis, hepatocellular deficiency and renal oligo anuric deficiency (Debetto & Luciani, 1988; Baruthio, 1992). Baruthio (1992) concluded that moderately soluble hexavalent compounds are carcinogenic, whereas highly soluble hexavalent compounds were quickly eliminated and did not cause cancer, but are implicated as mutagens.

1.1.1.3 Nickel

Nickel is used in electroplating, is a component of many alloys (for its corrosion resistance, high strength and durability) and in coinage. Nickel intake of 200-300 μg/day is adequate for human metabolism, it is implicated in the absorption of iron and thus blood formation (Moore & Ramamoorthy, 1984 b). Elevated levels of nickel have been highlighted as harmful to the environment. There is evidence of nickel carcinogenicity (Volesky, 1990 a), although this is largely associated with nickel within airborne particulates (Moore & Ramamoorthy, 1984 b). When nickel is

administered in acute excess it can interfere with the detoxification activities of the liver, and it has also been shown to produce some teratogenic effects in mammals (Moore & Ramamoorthy, 1984 b).

1.1.1.4 Mercury

Mercury and lead are also accumulated readily by organic matter (Vallee & Ulmer, 1972). Mercury salts from industrial effluents, mainly from the production of chlorine for bleaching paper, are converted to toxic methyl and dimethyl mercury by anaerobic bacteria in the sediments of water courses, thus entering the food chain (Volesky, 1990 a). In humans mercury will pass from the bloodstream and accumulate in the cerebellum and brain cortex causing symptoms of numbness, awkward gait and blurred vision (Volesky, 1990 a). The rise of environmental mercury levels means that they are tending towards the safe limit (the World Health Organization proposed acceptable daily intake is 0.3 mg Hg; WHO (1993)) and so man's addition to the load is of critical importance (Kirk & Lester, 1984; Volesky, 1990 a).

1.1.1.5 Lead

Inorganic lead (Pb^{2+}) is a metabolic poison and enzyme inhibitor, and can replace calcium in bones, remaining there as a reservoir for long term release. Organic lead (tetra ethyl and tetra methyl lead) is yet more toxic. The natural levels of lead in human blood are already close to that which is considered a reasonable toxicological limit (different authorities suggest safety levels in the range 0.2-0.8 ppm), leaving no safe margin for further exposure (Volesky, 1990 a).

1.1.2 Environmetal metal limits

The table below (Table 1.1) presents some of the data regarding those toxic metals produced by industrial activity which are of particular environmental concern and may be found in water. The European Economic Community legislation for metal limits in water intended for human consumption is shown [(1)] as maximum admissible concentrations (EEC, 1980), a previous directive (Directive 75/440/EEC) laid down the standards for surface water intended for the abstraction of drinking water (EEC, 1975). The WHO advice upon metal limits in potable water [(2)] demonstrate that

certain of the metals are regarded as a greater risk to health by this authority reflected in the lower limit (WHO, 1993). The concentration of metals routinely found in an untreated municipal sewage [3] is included in the table to afford a comparison with the potable water criteria (Lester, 1981). It can be seen from this example that many of the target metals in raw sewage may exceed the permitted levels for drinking water, however it is likely that the non-specialist treatment which it receives will reduce this metal load in line with the legislation. Indeed it is the industrial activities detailed in Section 1.1.1 which add the greatest metal load to the water system.

Table 1.1 Legislative limits and sample data for the metal content of water

Metal	EC potable water[1] (μg/l)	WHO potable water[2] (μg/l)	Raw municipal sewage[3] (μg/l)
Cd	5	3	6
Cr	50	50	290
Cu	3000	2000	310
Hg	1	1	7
Ni	50	20	330
Pb	50	10	230
Zn	5000	3000	2400

The maximum admissible concentrations for potable water and abstraction waters do not readily translate into discharge limits for industrial effluents. There are fears that the cost of compliance with more and more stringent criteria will be too much for industry to bear, whilst providing little extra in environmental benefits. This is because methodologies employed by the controlling authorites have not calculated the fraction of the heavy metal discharge which is bioavailable. There is often further difficulty in extrapolating toxicological data from animals to man (Kirk & Lester, 1984). Environmental agencies around the globe are now in consultation with science and industry to set reasonable limits which will be attainable by industry whilst still achieving the anti-pollution goals in the wider environment. The permitted levels for industrial discharge of metal must reflect the toxicity of the metal, its bioavailability

(speciation and solubility), and the other components of the discharge and receiving water which may complex the metal, so reducing its availability (Hall *et al.*, 1992).

It is evident that safe thresholds of toxic metal exposure may be exceeded through relatively small additions to the natural environmental metal load (Kirk & Lester, 1984). Now that many of the toxic health effects have been documented it is important that stringent measures are taken to ensure that the 'life-cycles' of toxic metals are identified and attempts made to reduce the additions of waste which would likely tip the balance of safety.

1.2 Waste clean-up legislation

In response to the growing body of information on the adverse effects of metal pollution, governmental authorities have begun to legislate in order to reduce the discharge of toxic industrial wastes. It is this legislation, the finite high-grade resources and the rising prices of raw materials (partly a result of primary producers passing on the cost of their own environmental clean-up) which has lead industries to reduce the amount of waste produced.

The range of regulations includes the 'duty of care' which is part of the Environmental Protection Act 1990 (Department of the Environment, 1991), and holds that the responsibility for the safe handling and disposal of waste rests with the producer. Operators of polluting processes are required to obtain authorization to continue operation and then to use the best available technology not entailing excessive cost (BATNEEC) to prevent or minimize pollution (Mabb, 1993).

The discharge limits for individual polluting concerns are specific. In the U.K. limits may be set by local water authorities if the discharge is to be very low, and can often be released into sewers. Discharge consents for processes with greater polluting capacity are granted by regional environmental agencies, following a full assessment of the process, the receiving water and the status of the local environment regarding pollution.

1.2.1 Waste minimization

Introduction of the Environmental Protection Act has forced industries to change their perspective on waste management and emphasis has been placed on the complete elimination of waste. Since this remains an unachievable goal, the priority has been reduction or avoidance of waste at source through changes in industrial processes and recycling before treatment by detoxification and disposal are considered (Crittenden & Kolaczkowski, 1993; Humphrey & Keller, 1997 a). Methods employed in toxic waste minimization may include:

- *separation* of the toxic from other harmless components of the waste stream;
- *conversion* of compounds to less toxic forms, e.g. by chemical reduction;
- *concentration* of dilute streams in order to recover and recycle metals;
- *improvement* of plant design or manufacturing process to consider an integrated approach including recovery and process recycle features.

The imposition of further directives has served to push the current equilibrium towards a minimization approach. The 'Protection of Groundwater' Directive (EEC, 1975) effectively curtailed the concept of 'dilute and disperse' leachate management for new landfill sites. It also required that by 1986 all discharges should have complied, including landfills which pre-dated the directive. As a consequence, economic technology to deal with the retrospective clean-up is urgently required in order to comply with legislation. Furthermore, some European countries are pressing for landfill to be classed as a disposal method of last resort (or at least for the collection of landfill gas and leachate to be imposed), with insurance against environmental impairment or a levy on operators (Mabb, 1993). As landfill costs rise, recycling becomes a more viable proposition and industry is encouraged to deal with its waste. The increasing demand for precious or strategic metals and the diminishing resources from geopolitically unstable suppliers, also combine to provide a powerful stimulus for the development of recovery methods (Volesky, 1987).

1.3 Metal removal technology

Industry is keen to comply with the standards imposed to safeguard their interests, whilst minimizing the cost so incurred. Technology is required which is both efficient and economic for a broad range of applications, since the industries producing metal wastes are of such a diverse nature. Current methods for the removal of toxic metals from solution include chemical precipitation and reduction (Cushnie, 1984), filtration and membrane technology, ion exchange and adsorption (Bolto & Pawlowski, 1987), electrochemical treatment (Norberg & Persson, 1984), and evaporative recovery (Nemerow, 1978).

1.3.1 The scope of existing technology

Removal, concentration and recovery from waste with a high concentration of impurity can yield large quantites of the metal, and is desirable, particularly when the metal is expensive, of strategic interest or easily fed back into the process. When single metal, concentrated waste streams of high value are to be treated, those techniques of recovery requiring equipment of high capital cost or expensive consumables such as precipitation, ion exchange or evaporative recovery can be efficient and cost effective. Such technology becomes too expensive at low metal concentrations, for example between 1 and 100 mg/l of dissolved metal (Volesky, 1990 b), or mixed streams of varying pH, carrying particulates. These wastes may also require defining by analysis before pre-treatments can be undertaken so as to prepare for the treatment process; the techniques of precipitation and neutralization, for example, demand use of chemicals specific to the waste. Since many of the waste-streams produced by industry fall into the latter, more complex state, their recovery by existing technologies may not be cost effective, and the recovery is driven solely by the threat of toxicity, and the legislation which is now in place. Industry seeks a complete treatment which is both effective and economical.

1.3.2 Adsorption technology, activated carbon

Adsorption technology is already a ubiquitous method for the treatment of waste-waters containing organics, and for potable water. Porous carbons have been used for

adsorption for many centuries. Early descriptions by the Ancient Egyptians in 1550 B.C., and later by Hippocrates and Pliny the Elder were of the medicinal importance of carbons. This involved batch use of powdered carbon, superseded by granular adsorbents only in the nineteenth century when bone char was used in the sugar industry (Rice & Robson, 1982). Activated carbon has emerged, since the 1960s, as one of the most effective methods of removing organic substances, both synthetic and natural, which occur in natural waters. Carbon is now used extensively in domestic appliances, throughout industry for separations, and in the municipal water industry for treatment of waste-water and supply of potable water to the consumer (Faust & Aly, 1987 a).

Adsorption is the phenomenon of concentration of a component at an interface rather than free in the bulk solution (Puri, 1970). The surface nature of activated carbon, its porosity, charge and hardness suit it to adsorption of organics from water when operated as a column filter, and whilst widely used in this manner, it is not considered an effective metal sorbent. Much work has been done in the past to improve the uptake capacity and specificity of carbon for organics. Research into the source materials for granular activated carbon (GAC), the activation process and chemical pre-treatments has increased the surface area and altered the carbon's surface complexes with the effect of improving its uptake characteristics. Tailoring of the hardness of carbon granules and the bulk density has improved performance in column operation, however innovation into the range of impurities which may be adsorbed by carbon, beyond the adsorption of organics, has lagged behind.

1.3.2.1 Co-adsorption

Metals are not thought to be taken up by carbon in significant amounts, and so a complete waste-water treatment solely by use of activated carbon is not effective. In order to provide a complete treatment with activated carbon, taking full advantage of a system which can adsorb complex mixtures of organics and filter out particulates whilst utilizing existing infrastructure and technology, a co-adsorbent suited to the uptake of metals has been investigated.

1.3.2.2 Biological adsorption

The adsorption (or concentration effect) of metals by micro-organisms is a phenomenon which has long interested microbiologists and has been recognized more recently by biotechnologists for its potential in the remediation of waste-waters contaminated with toxic heavy metals (Muraleedharan *et al.*, 1991). The adsorption of toxic pollutants by lower plants, animals and micro-organisms is a phenomenon already used as a biological indicator of pollution (lichens are a prime example). High concentrations of pollutants within the organisms can be indicative of contamination within the environment, as can a sudden decline in numbers of a usually healthy population which is susceptible to pollution. The combination of an activated carbon filter with a biofilm co-adsorbent of micro-organisms, which can accumulate metals without adverse effects, may exploit the strengths of each adsorbing system to provide the complete waste-water treatment package, long sought after by carbon companies who acknowledge the limitations of carbon adsorption.

1.3.3 Target sites: dumpsite remediation

Environmental organizations have focused upon heavy metal pollution because these contaminants are pernicious and persistent. Chromium, one of the metals studied in this research, is one of the most common metal contaminants in US soils (Weng *et al.*, 1994). A niche of particular importance and of interest to carbon producers developing metal decontamination and environmental remediation techniques are the toxic metal dumpsites (Smith & Weber, 1990).

Many dumpsites were created when discharge limits were imposed but while treatment technology was still not widely in use. During this time waste treatment amounted to barreling and removal to dumpsites where it would be buried. Such barrels could contain undefined mixtures of toxic metals and organics dumped without prior effort to reduce the toxicity. In many cases corrosion of these containers and the leaching effect of rainfall has caused contamination of run-off waters and the water-table. Waste-water such as this, containing dilute mixtures of toxic components and particulates is especially problematic to many of the available technologies, and so inexpensive, robust, 'low tech' approach to purification is sought by use of biofilm coated carbons.

Literature Review

2.1 Conventional metal removal treatments

Physico-chemical waste-water systems can accomplish good treatment levels but can often be energy and operating cost intensive (Ying & Weber, 1979; Sharma & Forster, 1993). Humphrey & Keller (1997 a) however, contend that the operating costs of competing technologies are comparable and that it is the capital costs that vary more. Adsorption by activated carbon is the most widely applicable and often most cost-effective of the physico-chemical systems (Ying & Weber, 1979).

2.1.1 Chemical precipitation

Chemical precipitation is widely used for industrial waste-water treatment (Edwards, 1995). Generally alkali must be added to the waste to bring the pH to above 8.5 or 9, at which the metals begin to precipitate. An homogenous and efficient reaction is gained through mixing. Treatment of dilute waste tends to be more effective than concentrated streams (Edwards, 1995). Further treatment includes use of cationic polymers (most effective at neutral or slightly acid pH), and anionic polymers (effective in basic solutions) to coagulate the tiny particles formed by precipitation. The larger the particles the quicker they will settle out (Edwards, 1995).

Chromium waste is generally reduced from the hexavalent to trivalent state with the strong reducing agents, bisulphite or sulphur dioxide, followed by precipitation of $Cr(OH_3)$ at alkaline pH before settling out (Huang & Bowers, 1978). The hydroxide is highly hydrated and may contain up to 80% water (by volume) making its disposal expensive (Kim & Zoltek, 1977).

The primary disadvantage is that additional solids are generated by the addition of chemicals during treatment; there is a large volume of sludge to then be disposed of (Edwards, 1995). Dilute wastes are better treated than concentrated waste, and a batch treatment, which may not be suitable for all applications, is recommended. The waste must be carefully defined to ensure appropriate use of chemicals (Cushnie, 1984), and the pH charted to utilize the coagulating agents at their optimum (Edwards, 1995). A further consideration is the need for large settling tanks, when land may not always be available for this (Alaerts *et al.*, 1989).

2.1.2 Membrane filtration

Membrane based separation processes are effected by selectively passing one or more components of a stream through the pores of a membrane (the permeate) whilst retarding the passage of others (the retentate) (Humphrey & Keller, 1997 b).

There is an array of membrane technology including the pressure driven separations; reverse osmosis, ultrafiltration, and microfiltration, and the concentration or electrical gradient driven separations of ion exchange, and electrodialysis respectively (Coulson *et al.*, 1991; Porter, 1997). The membrane processes are grouped into these classifications by the size of components which they are able to retain;

- electrodialysis < 5 nm
- reverse osmosis < 5 nm
- ultrafiltration 0.1 μm-5 nm
- microfiltration 10-0.1 μm.

The large pore size of microfiltration membranes means that the process is not suitable for the removal of metals from solution. Ultrafiltration is not suitable for all metal bearing wastes; copper and zinc from copper-plating rinsewater may pass through the membrane (Edwards, 1995), but is commonly used for removal of contaminated oil from metal-finishing bath wastes (Porter, 1997). Reverse osmosis is the pressure driven transfer of water through a membrane against the osmotic potential, i.e. removing water from the side with a higher solute concentration (Coulson *et al.*, 1991). The metals most commonly encountered in metal-plating wastes, chromium, copper, cadmium, zinc, tin and nickel, may be treated by reverse osmosis. Waste is minimized since the purified permeate is recycled back to the rinse line and the concentrated metal back the plating tank (Porter, 1997).

Although useful in some applications the technologies of ultrafiltration and reverse osmosis are not robust enough to deal with waste streams containing particulates. The tiny size of the pores causes the membranes to become fouled very quickly, although backflushing/reversing flow or cross flow operation can help to prevent this. Membranes are also sensitive to pH. The pH of many rinsewaters will accelerate the hydrolysis of cellulose acetate membranes. Cellulose acetate membranes are most

resistant to hydrolysis at pHs of 3-8, yet for example, the pH of zinc cyanide plating waste cannot be lowered to less than 9 without metal precipitation (Porter, 1997).

Membrane equipment is modular and relatively cheap to buy, commission and run, although the membranes themselves are expensive and so are best reserved for high value products (Edwards, 1995).

2.1.3 Electrochemical processes

Electrolytic processes may be used to remove metals and are already employed for metal finishing rinsewaters. Metals are deposited onto the cathode of an electrolytic cell (electrowinning cell). Some cells incorporate membranes through which the metal (for example Cr^{6+}) is concentrated for recovery.

Electrolysis requires a long contact time since the process of deposition is slow, and so it is generally incorporated into a recirculating system to allow for this. Electrolysis of dilute streams requires an electrode of large surface area due to the inherent process inefficiency. Waste-waters should be without biological material and particulates since these may coat the electrode reducing efficiency, or may themselves collect metal before sloughing into the discharge water (Edwards, 1995).

2.1.4 Evaporative recovery

Evaporation is the removal of water from a solution by boiling. By use of a suitable vessel, an evaporator, the vapour may be withdrawn and the metal concentrated in the remaining liquor (Coulson *et al.*, 1991). The vessel must be heated to provide the latent heat of vaporisation to the solvent. The process is made more energy efficient by recovery of heat from the vapour. Evaporative technology is already used to concentrate rinsewaters from metal finishing, and can be employed to further concentrate residue produced by other treatment processes (Edwards, 1995).

Special consideration is required if components of the waste are inversely soluble since this results in scale deposition on the heating surface (Coulson *et al.*, 1991) reducing the process efficiency (Edwards, 1995). Problems encountered include foaming, necessitating addition of antifoams or use of mechanical methods to break

up the foam, adding to the cost of the process. Equipment must be carefully controlled to avoid carry-over of liquid in the vapour by entrainment (Edwards, 1995). Waste recovered by the process of evaporative recovery will be a concentrated solution but remains as a mix of components. The process is relatively expensive in capital costs and fuel and is unlikely to be economical unless the recovered waste has intrinsic value (Nemerow, 1978).

2.1.5 Adsorption

In adsorption, molecules distribute themselves between two phases, one a solid, the other a liquid or gas (Coulson *et al.*, 1991). Adsorbed molecules diffuse from the bulk of the fluid to the surface of the solid adsorbent. Separation of waste components from solution depends upon these components being more readily adsorbed from the bulk than the solvent. The adsorbent may take the form of molecular sieve (zeolite), activated carbon, silica gel or activated alumina (Coulson *et al.*, 1991), although some low technology filtration systems such as trickle reed beds are in effect also acting as adsorbents. Silica gel and activated alumina are hydrophilic and are used for water adsorption (Coulson *et al.*, 1991). Molecular sieves and activated carbon will readily adsorb organics and are effective for waste-water treatment. The lattice structure of molecular sieve can be synthesized with precise dimensions so excluding larger molecules, whilst adsorbing the smaller target molecules specifically (Coulson *et al.*, 1991).

A disadvantage of molecular sieve adsorption is that the sieve matrix can be expensive, such that adsorbates of low value may not be economical to treat. It may also be too specific for mixed waste streams. Columns suffer from fouling by particulates and so a pre-filter is advised, and the zeolite is prone to high attrition rates. The cost of regeneration of the sieve must be included in the capital and running costs of the process since the matrix has a finite adsorbing capacity (Edwards, 1995).

Adsorption by activated carbon is often the cheapest mode of separation (Hutchin, 1997). Activated carbon is widely used in the treatment of waste-water and for potable water. This is discussed further in Section 2.2.

2.1.6 Ion exchange adsorption

Ion exchange resins may be used to treat waste-water containing soluble metals which is free from particulates; the technology is already employed in waste metal recovery from metal finishing processes (Edwards, 1995). The basis of the technique is to separate ionic species; the rate at which ions diffuse between exchanger and liquid is determined by the concentration gradient between the phases and the requirement for maintaining their electroneutrality (Coulson *et al.*, 1991). Cation or anion exchangers are available depending upon the application; the resin is ordinarily an organic polymer network with attached ionic functional groups (Anderson, 1997). The adsorbed metals must be removed from the resin by regeneration, but this means that they can potentially be reused.

Ion exchange is not an effective process for mixed waste streams as chelating and complexing agents may inhibit the ion exchange (Kim & Zoltek, 1977; Edwards, 1995). The pH of mixed streams can vary widely affecting adsorption onto the matrix (Edwards, 1995). Kim & Zoltek (1977) noted that flow rates must be carefully controlled for efficient chromium removal. Ion exchange resins are also susceptible to fouling by organics and some inorganic materials and can become blocked by particulates (Edwards, 1995). Ion exchange media may be too specific for wastes which are not fully characterized and are an expensive choice (Alaerts *et al.*, 1989), although for single component wastes of a high value they may be useful if the metal is to be recovered and reused.

2.1.7 Competing novel technologies and alternative adsorbents

There are many different forms of biosorbent championed by authors in the field of research using; sphagnum moss peat (Sharma & Forster, 1993; Low *et al.*, 1997), algae (Kuyucak & Volesky, 1990), cyanobacteria (Garnham & Green, 1995), fungi (Sag & Kutzal, 1996) and yeast (Rapoport & Muter, 1995).

Sharma & Forster (1994 a) also investigated Cr^{6+} adsorption by alternative low cost adsorbents by screening the potential of sawdust, dried sugar beet pulp, bagasse, maize cob and, leaf mould (Sharma & Forster, 1994 b) in batch sorption tests. Uptakes were described by the Langmuir isotherm. Other authors have investigated

the potential of: alginate beads (Araujo & Teixeira, 1997), coir (Quek *et al.*, 1998), coconut husk and palm pressed fibres (Tan *et al.*, 1993). It is clear that there is a broad array of alternative sorbents and biosorbents, many still in the research stages, although some are already commercially available. An algal biosorbent AlgaSORB™ and a granulated bacterial biomass AMT-BIOCLAIM™ have been developed for treatment of waste-water (Kuyucak, 1990).

2.2 Activated carbon

Porous carbons are obtained as a residue after the volatile components of a carbonaceous material are removed by a thermal process in the absence of air, leaving the graphite-like plates called basal planes or layer planes. This is the first stage in the production of activated carbon and is known as pyrolysis and carbonization (Byrne & Marsh, 1995). The starting materials are naturally occurring and can include lignite or bituminous coal, peat, wood or nutshell.

2.2.1 Manufacture and structure of activated carbon

Activated carbon is produced by further treatment of the carbonized material resulting in an extensive internal pore structure and a surface area characteristically between 500 and 1,500 m^2/g. This may be achieved by physical or chemical activation. Physical, steam or carbon dioxide activation at temperatures between 800 °C and 1,000 °C removes some carbon atoms by gasification. Water molecules are small, and so steam activation is preferred since the water molecules can penetrate faster and deeper into the porous carbon network compared with the larger carbon dioxide molecules (Byrne & Marsh, 1995). The layer planes possess a range of susceptibilities to the gasification reaction, some planes are removed by the process while others are left behind so opening up the pore structure in a random fashion. Generally the slower the reaction, the more extensive is the development of porosity (Byrne & Marsh, 1995).

Chemical activation involves incorporation of inorganic additives (e.g. $ZnCl_2$, phosphoric acid, sulphuric acid) prior to carbonization, which then proceeds at a

much lower temperature, typically 500 °C (McEnaney, 1988). Chemical activation swells the raw material opening up the structure. On carbonization the chemical acts as a support and does not allow the resulting char to shrink. The activation agent is generally removed by washing.

The low temperature of chemical activation means that layer planes are not found, rather each particle is a partly aromatic, partly aliphatic organic molecule or highly cross-linked polymer. Unlike steam activated carbons the pore walls are not flat but rough, and the carbon rather less hydrophobic. It is the enhanced surface area and the nature of that surface, dependant upon the starting material and chemicals in the activation process, which drives the adsorption. The hazardous chemicals used for chemical activation are subject to pollution regulations and hence physical activation is likely to be favoured over chemical activation in the future (Byrne & Marsh, 1995).

The pore structure is dictated by the raw materials and the activation process. Pores may be divided into three groups; macropores, mesopores, and micropores, corresponding to their size, although the delineation is arbitrary. Pores are divided thus, by recommendation of the International Union of Pure & Applied Chemistry (IUPAC) (McEnaney, 1988):

- macropores; diameter > 50 nm
- mesopores; diameter 2-50 nm
- micropores; diameter < 2 nm

The macropores of granular activated carbon arise from the structure of the starting material, whether it be natural or man-made. Some meso- and macroporosity may also be created by shrinkage during carbonization (Byrne & Marsh, 1995). Carbon of a botanical origin (wood or nutshell) will maintain some of its original cell structure. The younger fossil fuels (peat) can show structure which relates to it in life. Compression (briquetting or extrusion) of finely milled carbon, will lead to a man-made macroporous structure. The void space remaining between the powder particles constitutes the macropores. Powdered activated carbons are not generally rich in macropores due to the small particle size.

Only a tiny fraction of a carbon's adsorbing surface area is supplied by the macropores, rather these act as transport pores for molecules to reach the interior of carbon granule. The adsorption process is distinctly mesoporous and microporous in nature (Byrne & Marsh, 1995).

Mesopores and micropores are produced through the activation process. The microporous structure of the carbons consists of a tangled network of defective carbon layer planes. Functional groups (such as C-OH and C=O) are bound to the periphery of the layer planes, and these exert an influence on adsorption (Puri, 1970; Faust & Aly, 1987 c). The planes may typically be 5 nm wide, cross linked by aliphatic bridging groups to form small stacks. The stacks (characteristically of two, three or four planes) have variable interlayer spacing within the range 0.34 to 0.8 nm, and so it is these that supply much of the enhanced surface area of the carbon.

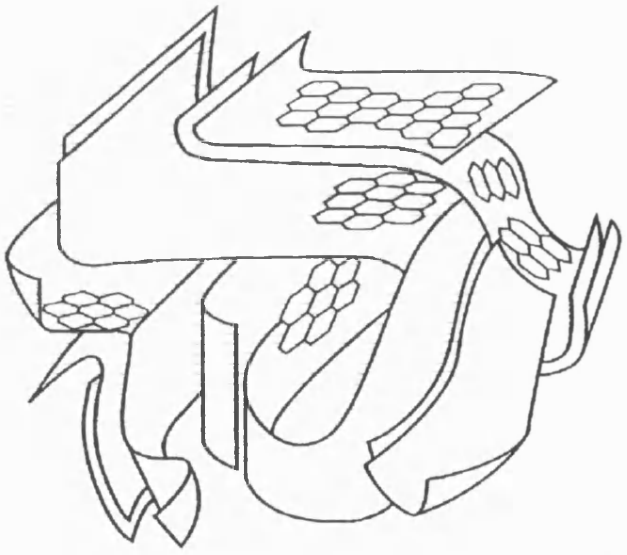

Figure 2.1 A schematic diagram of activated carbon (Dussert, 1994)

McEnaney (1988) further divides the micropores into *ultramicropores,* of enhanced adsorption potential, and *supermicropores,* the wider pores of up to 2 nm width. The greater fraction of surface area is derived from the micropore and mesopore regions, and these are most important in gas and liquid phase adsorption respectively (McEnaney, 1988). Activated carbon from nutshell is rich in micropores, coal based carbons possess a wider range of mesopores, and wood or peat, an extensive

macropore structure. Carbon microporosity in the form of slit shaped pores can be composed of the spaces between carbon layer planes and gaps between stacks. These pores can therfore be much smaller than the arbitrarily defined upper limit of 2 nm for micropore width (McEnaney, 1988). Constrictions in the micropore structure may limit access to much of the carbon's porosity. A carbon with a microporous structure, but with mesopores and transport pores to deliver the adsorbate into the network will provide the most efficient use of the enhanced surface.

2.2.2 The nature of adsorption

Adsorption may be by 'physisorption' which arises from dispersion forces (van der Waals or London forces), or by 'chemisorption' through the exchange or sharing of electrons, so giving rise to ionic or covalent bonds. Adsorption allows the surface molecules of the carbon to achieve a balanced state. The more disordered, single, unstacked graphite-like layers that there are in a carbon, the better it is for chemisorption. At the same time there are many exposed defects, dislocations and discontinuities in the layer planes of microcrystalline carbons apart from the edges of the carbon layers (Puri, 1970). These are called 'active sites', associated with high concentrations of electron spin centre and therefore expected to play a significant role in chemisorption. Chemisorption of elements such as oxygen, hydrogen, nitrogen, halogens and sulphur on the carbon surface gives rise to stable compounds called surface complexes (Puri, 1970).

Oxygen is chemisorbed more readily than many other elements, and carbon-oxygen complexes are by far the most important in influencing the surface reactions, surface behaviour, wettability and catalytic properties of carbons (Puri, 1970). All types of carbon are covered with oxygen complexes unless special care is taken to eliminate them (Faust & Aly, 1987 c). There is debate over the exact nature of surface oxides. Puri (1970) suggested that the combined oxygen is present mainly at the edges of giant molecules which constitute the main adsorbing surface and so the oxygen complexes exercise a considerable influence on the surface behaviour and surface reactions of carbons.

2.2.3 Characterization of adsorption/modelling

The efficacy of a carbon's adsorption can be presented graphically. The representation of the amount of solute adsorbed per unit of adsorbent as a function of the equilibrium concentration in bulk solution at constant temperature, is termed the adsorption isotherm. The shape of the isotherm can give qualitative information about the adsorption process and the surface coverage by the adsorbate (Faust & Aly, 1987 b). There are many adsorption models including the Freundlich, Langmuir, BET, and Linear Adsorption models, although the Freundlich and Langmuir models are most commonly used for describing and comparing adsorption processes.

2.2.3.1 Freundlich Isotherm

The Freundlich isotherm is routinely used in aqueous systems and is a standard throughout the carbon industry (Hamerlinck, 1994). It is an empirical mathematical model expressed thus:

$$\frac{x}{m} = KC_e^{1/n}$$

where: $\frac{x}{m}$ = loading (mass of solute adsorbed per unit mass of adsorbent) (mg/g),

C_e = equilibrium concentration of the solute after adsorption (mg/l),

K and $1/n$ are constants characteristic of the system.

The data is linearized by taking logarithms:

$$\log\frac{x}{m} = \log K + \frac{1}{n}\log C_e$$

Plotting log x/m against log C_e gives a straight line of slope 1/n and intercept K, at C_e=1. This is demonstrated by the log-log plot in Figure 2.2

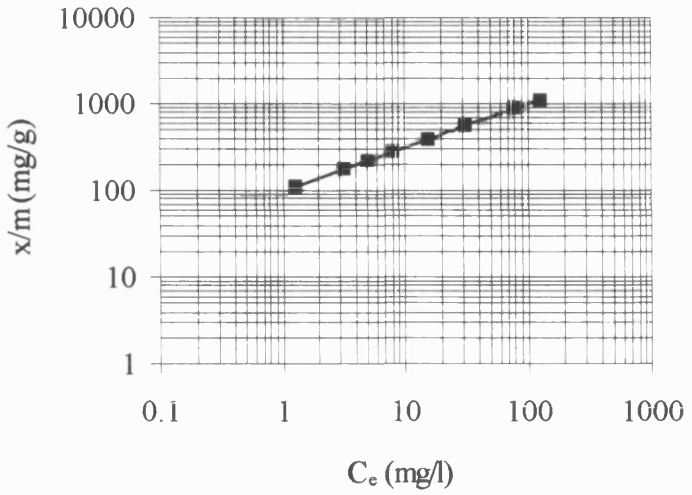

Figure 2.2 The logarithmic form of the Freundlich adsorption isotherm.

2.2.3.2 Langmuir Isotherm

The Langmuir model, which may also be called the *ideal localized monolayer model* is based on several assumptions;

- molecules are adsorbed on specific sites on the adsorbent surface,
- a monolayer is formed i.e. only one molecule is found at each site,
- the geometry of the site dictates its area, which does not change,
- the adsorption energy is the same at all sites.

The Langmuir model is routinely applied to adsorption from the gas phase but may also be used for liquid phase applications (Coulson *et al.*, 1991). For adsorption from solution by solid adsorbents, the Langmuir adsorption isotherm is expressed as:

$$X = \frac{X_m b C_e}{1 + b C_e}$$

where: X = loading, x/m (mg/g),

C_e = equilibrium concentration of the solute (mg/l),

X_m = loading required for monolayer coverage of the surface (mg/g),

b = constant related to the heat of adsorption.

2.2.3.3 BET Isotherm

The BET isotherm was developed by Brunmauer, Emmett and Teller. It is a generalization of the Langmuir model of ideal localized monolayer which accounts for multilayer adsorption (Faust & Aly, 1987 b).

$$X = \frac{X_m BC}{(C_s - C_e)\left[1 + (B-1)C_e/C_s\right]}$$

where: X = loading (mg/g),

C_e = equilibrium concentration of the solute (mg/l),

X_m = loading required for monolayer coverage of the surface (mg/g),

C_s = solubility of the solute in water at a specified temperature (mg/l),

B = constant.

The BET isotherm is used to estimate the total surface area of a carbon, determined by nitrogen or butane gas. This parameter is used for comparing the adsorption capacities, and in selecting a carbon appropriate to the adsorbate, although often not all of the surface area is accessible to the molecules to be adsorbed. Estimates of surface area made by this method can be unrealistically high when applied to microporous carbons since adsorption in micropores does not take place by successive build-up of molecular layers (McEnaney, 1988).

2.2.3.4 Other Isotherms

The simplest of isotherms is the Linear Adsorption Isotherm, or Henry's Law (Faust & Aly, 1987 b). The amount adsorbed varies directly with the equilibrium concentration of the solute:

$$X = K_h C_e$$

where: X = loading (mg/g),

C_e = equilibrium concentration (mg/l),

K_h = constant.

The isotherm is obtained under conditions of low solute concentration. The adsorbed layer is extremely dilute and the amount adsorbed is only a fraction of the monolayer capacity. Almost all the adsorption isotherms are reduced to Henry's Law at low concentrations. (Faust & Aly, 1987 a)

2.2.4 Adsorption dynamics

The adsorption of substances to the carbon surface is a complex process. The simplified dynamics were described by Faust & Aly (1987 b) as a three stage process where the solute;

i) transfers from bulk solution through the liquid film to the exterior surface of the carbon,
ii) diffuses into pores of the adsorbent except for the tiny proportion adsorbed on to the external surface,
iii) and is adsorbed on the interior surfaces of the pores and capillary spaces of the carbon.

The rate determining step is the diffusion through the surface film, the last step is an equilibrium reaction. Many factors can influence the adsorptive capacity:

- *adsorbate properties;* group functionality, branching geometry, polarity, hydrophobicity, dipole moment, molecular weight and size, and aqueous solubility,
- *solution conditions;* pH, temperature, adsorbate concentration, ionic strength, and competitive solutes,
- *adsorbent properties;* surface area, pore size and distribution, distribution of functional groups, and ash content.

2.2.5 Metal adsorption by activated carbon

The chemical nature of the carbon surface as well as the target metal ions are of great significance to the adsorption process (Budinova *et al.*, 1994). In early work it was suggested that the process of adsorption is simply through provision of an enhanced surface area with a plethora of oxygen functional groups (Puri, 1970). Corapcioglu &

Huang (1987 a) concur that surface functional groups and acidity are important in adsorption of inorganic components such as metals to activated carbon. Ibrado & Fuerstenau (1992) agreed that the nature of the carbon and more specifically its structure are important but through adsorption of gold cyanide to a series of carbonaceous adsorbents, they contend that there is a stronger correlation with the degree of graphitization. These authors suggest that the majority of adsorption occurred on the plate faces of the graphite crystallites of activated carbon (Ibrado & Fuerstenau, 1992). Diamadopoulos *et al.* (1992) related increased ash content of activated carbon to enhanced arsenic (V) uptake. Carbons with high ash content were able to remove up to 5 times more arsenic than carbon with no ash, and differences of surface area were of secondary importance. This was due to interaction with the metal oxides and metal ions which constitute a significant fraction of the ash (Diamadopoulos *et al.*, 1992). Mostafa (1997) claimed that the pH and surface chemistry of an activated carbon made from rice husks was more important in the removal of mercury, lead and cadmium ions from solution than was its enhanced surface area.

H-type and L-type carbons

There is some little confusion in the literature regarding classification of carbon by surface acidity and the groups that each of the carbon types will adsorb, however this may simply be due to a conflict in use of terminology. Faust & Aly (1987 c) have noted the definitions for the categorization of carbons as H- and L-type carbons. Carbons activated at high temperature, generally in steam, are H-type carbons (Huang & Bowers, 1978) which adsorb H^+ ions (Faust & Aly, 1987 c). A paper produced by Huang & Bowers (1978) is at variance with others on this matter, for although in agreement that an H-carbon raises the pH of water through a hydrolytic reaction, they state that the carbon adsorbs only *bases*. Low temperature activated carbons are L-type carbons which primarily adsorb OH^- ions (Faust & Aly, 1987 c). The addition of water to carbon results in a hydrolytic reaction, the H^+ ions are adsorbed to the carbon surface and hydroxyl ions evolved, thus causing the high pH of the bulk (Huang & Wu, 1975). In neutral or alkaline pH surface oxygen complexes oxidized water molecules at the carbon surface thus;

$$C_xO + H_2O \Leftrightarrow C_x^{2+} + 2\,OH^-$$

or $$C_xO_2 + H_2O \Leftrightarrow C_xO^{2+} + 2\,OH^-$$

It becomes apparent from the literature that a definitive explanation of the events resulting from the simple action of adding carbon to water is not available; many authors have described the reactions in terms expedient to their hypothesis for the adsorption mechanism. The reaction is complex and dependent upon many factors, not least the surface groups of the carbon and their status, the relative importance of which is hard to elucidate in what is a dynamic system.

Budinova *et al.* (1994) measured the pH of 3 carbons from different raw materials; apricot stones, coconut shell, and a more conventional, lignite coal. They stated that the high pH (i.e. 9-10) of water after 48 hours contact time reflected the predominance of basic functional groups, classing them as H-type carbons. The effect of pH on adsorption was marked; Pb^{2+}, Zn^{2+}, Cu^{2+} and Co^{2+} adsorption was negligible at pH below 2.0 (Budinova *et al.*, 1994). Adsorption increased with increasing pH, reaching a maximum at between pH 4.0 and 6.0 when the metal ions replaced H^+ ions on the carbon surface. Copper adsorption by the lignite based carbon was poorer than the other carbons. Budinova and co-workers related this to a surface charge which was very much dependant upon the pH of the solution. In general adsorption by apricot carbon was better than coconut shell, which was in turn superior to lignite coal, and the amount of each metal ion adsorbed increased in the order $Pb^{2+} > Cu^{2+} > Zn^{2+} > Cd^{2+}$, independent of carbon type (Budinova *et al.*, 1994).

In general the adsorption of cationic metals increases abruptly at a specific pH (Corapcioglu & Huang, 1987 b). Almost 100% adsorption of anions e.g. CrO_4^{2-} may be achieved at low pH, this efficiency of removal decreasing as pH increases (Corapcioglu & Huang, 1987 b).

2.2.5.1 Chromium adsorption

Chromium removal by activated carbon is a particularly complex process. Huang & Wu (1977) noted that hexavalent chromium removal by activated carbon was pH dependent and in this early work found that chromium adsorption capacity reached a maximum at pH 6. The understanding of the phenomenon of Cr(VI) interaction with

carbon is further complicated since it involves reduction of the Cr^{6+} to Cr^{3+} in the presence of the carbon (F400) but with preferential Cr^{6+} adsorption. There is some evidence that reduction occurs only at the exterior of the carbon and not inside the pores where the solution chemistry is different from that of the bulk (Hart *et al.*, 1980).

The kinetics of the interfacial phenomenon is not fully elucidated (Huang & Bowers, 1978), but these authors do advise that the presence of Cr^{3+} has considerable detrimental effect upon an activated carbon process for hexavalent chromium adsorption. If Cr^{6+} should be reduced to Cr^{3+} the *total* chromium remaining in the effluent is likely to remain high since Cr^{3+} is not readily adsorbed by H-type carbons such as F400. There are still conflicting opinions over which is the optimum pH for treating chromium waste, and the relative importance of reduction and adsorption at different pHs. The mechanism of reduction also causes a significant increase in solution pH, thereby slowing the rate of Cr^{6+} adsorption. Alaerts *et al.* (1989) noted that both chromium removal mechanisms lead to an increase in solution pH, since adsorption involved an exchange with hydroxyl ions from the carbon surface, and reduction used protons with the resulting formation of water thus;

$$Cr_2O_7^{2-} + 14H^+ + 6e^- \Leftrightarrow 2Cr^{3+} + 7H_2O$$

Chromium solutions of 20, 50 and 100 mg/l at an original pH of 2.5 had equilibrium pHs of 3.2, 5.6 and 6.4 respectively, after exposure to carbon at a dosage of 5g/l (Alaerts *et al.*, 1989). This demonstrates that pH setting for experimental purposes is not a simple task; the final pH will be dependent upon the degree of adsorption, which is in turn partly a function of its pH.

The effect of pH upon chromium adsorption and reduction

Kim & Zoltek (1977) found that for batch operation a pH of between 3.4 and 4.0 was optimum for chromium removal from a 100 μM Cr^{6+} starting solution, but unlike Huang & Wu (1977) did not assess what fraction had been reduced to Cr^{3+}. Further experiments suggested that hexavalent chromium removal was optimum at pH 2.5, whilst Cr^{3+} was better removed at higher pH, i.e. ≥ pH 4.0. At pH 4.0 almost no Cr^{3+} was found in solution either because it was all adsorbed or indeed had not actually

been reduced from Cr^{6+} at that pH. Varying the pH of a Cr^{3+} solution (without Cr^{6+}) increased its adsorption but could also cause precipitation of the hydroxide (Kim & Zoltek, 1977). Bowers & Huang (1980) found Cr^{6+} adsorption was optimum at pH 2.5, and decreased at higher pHs due to decreasing electrostatic attraction between the positively charged carbon surface and the anionic Cr^{6+} in solution. Alaerts and co-workers (1989) found that the Cr^{6+} removal efficiency from a 20 mg/l starting concentration using 5 g/l of the coconut shell based carbon was almost 100 % at pHs up to 7.0. The relative contribution of reduction increased as pH decreased, until at pH 2.0 half of the Cr^{6+} removal was by adsorption, and half by reduction to trivalent chromium (Alaerts *et al.*, 1989).

At pH lower than 2.5 hexavalent chromium was rapidly reduced to cationic trivalent chromium which was not adsorbed by the carbon (Bowers & Huang, 1980). Bowers *et al.*, (1992) found that 90% of the Cr^{6+} in a solution was reduced at pH 2.0, and only 10% adsorbed by the carbon, but by applying a passive voltage to the system the reduction could be inhibited by up to 94% with a concomitant increase in adsorption.

In desorption studies chromium that desorbed at alkaline pH was hexavalent whilst acid desorption released trivalent chromium (Kim & Zoltek, 1977).

There is some disagreement as to whether the reduction of Cr^{6+} could be suppressed to improve chromium removal efficiency. In column operation Kim & Zoltek (1977) concluded that adsorption was almost pH independent providing that Cr^{6+} and proton concentration were equimolar. Further, they suggested that by this method or under a preponderance of $HCrO_4^-$ species any reduction could be minimized. Later work by Bowers and Huang (1980) could not replicate these findings.

Shashikanth & Shantha (1993) found that activated carbon derived from bagasse (solid waste from the sugar industry) was suited to Cr^{6+} adsorption, whether activated by physical or chemical means. They described adsorption by the Freundlich, Langmuir and BET isotherms, but only the Freundlich isotherm demonstrated any difference between the modes of activation. Chemically activated carbon was superior to physical activation only at the lowest impregnation ratio of 0.25, any increase in the use of activating agent lead to a decrease in chromium uptake. Alaerts and co-

workers (1989) used the Freundlich isotherm to describe the adsorption potential of a range of carbons. The capacity of a coconut shell based carbon was superior to both granular F400, and powdered activated carbons. These authors also noted that carbon dosage, carbon particle size and initial chromium concentration are all part of the concert of effects with a bearing upon the carbon adsorption capacity.

Chromium speciation

It is important to remember the complex solution chemistry of chromium compounds, since the chromium species found in bulk solution will affect the reactions occurring at the carbon adsorbing surface. In basic solutions above pH 6 CrO_3 forms the yellow chromate ion CrO_4^{2-} ; between pH 2 and 6, $HCrO_4^-$ and the orange-red dichromate ion $Cr_2O_7^{2-}$ are in equilibrium; and at pH values <1 the main species is H_2CrO_4 (Cotton & Wilkinson, 1988).

$$HCrO_4^- \Leftrightarrow CrO_4^{2-} + H^+$$

$$H_2CrO_4 \Leftrightarrow HCrO_4^- + H^+$$

$$Cr_2O_7^{2-} + H_2O \Leftrightarrow 2\,HCrO_4^-$$

The distribution of chromium (VI) species is not only dependent upon the pH, but also the total chromium (VI) concentration. The higher the concentration of chromium (VI) the higher the concentration of dichromate, but at very low Cr (VI) concentrations almost no dichromate is present (Shen-Yang & Ke-An, 1986).

Factors affecting the metal ion availability

Complexation of chromium, by a chelating agent such as EDTA, may lead to an alteration in its uptake by carbon. Gajghate and co-workers (1992) used a chromium diphenyl carbazide (CDC) complex in order to improve chromium sorption, although again it was found that the presence of Cr^{3+} decreases chromium removal even in the form of CDC. Due to a lack of experimental controls however the work did not compare directly the uptake of free chromium as opposed to complexed chromium. Factors other than the metal speciation which may affect the metal availability can be complexation by components of growth medium which act as ligands and precipitation through changes in the solution chemistry (Hughes & Poole, 1991).

2.2.5.2 Cadmium and nickel adsorption

Reed & Matsumoto (1993) modelled the adsorption of cadmium using Langmuir and Freundlich isotherms. They hypothesized that the cadmium removal from solution, which increased with increasing pH, was comprised of two components, adsorption and surface precipitation (Reed & Matsumoto, 1993). Marzal and co-workers also found that cadmium and zinc adsorption increased with increasing pH (Marzal *et al.*, 1996), and likewise copper and nickel (Seco *et al.*, 1997). Cadmium adsorption increased as the pH was raised until pH 9 when cadmium was precipitated as cadmium hydroxide (LeyvaRamos *et al.*, 1997). The adsorbed cadmium species is generally agreed to be Cd^{2+} (Huang & Wirth, 1981; LeyvaRamos *et al.*, 1997) and it is this and Ni^{2+} which are the prevailing species in the following experiments.

2.3 Biological metal sorption

All micro-organisms interact with metals; some metals are essential minerals required at trace levels for growth, whilst many are toxic, having no known biological function. Metal toxicity is largely as a result of denaturation of cell proteins (Gadd & Griffiths, 1978) since toxic metals form strong bonds with groups containing nitrogen and sulphur atoms (Remacle, 1990 b). The delicate equilibrium of metabolic necessity and cellular toxicity is balanced by the metal tolerance mechanisms of the cell. These mechanisms are specific not only to the organism and its physiological state but also the metal or metals involved, and the bathing menstrua; metal may be bound to organic materials or precipitated, complexed or subject to ionic interactions, each altering its biological availability and activity (Gadd & Griffiths, 1978). Texts devoted to metals and micro-organisms are available (Hughes & Poole, 1989; Volesky, 1990) and so only a limited overview is supplied here.

A cell's metal tolerance may be accomplished by a number of mechanisms reflecting an ability either to survive in a metal rich environment without internalizing that metal above a toxic level, or contrastingly ability to accumulate the metal and withstand high intercellular concentrations by immobilization, compartmentalization, precipitation or volatilization of the metal. Many of these mechanisms are genetically mediated (Section 2.3.1).

Categorization of metal-microbe interactions is largely an academic exercise since modes of resistance or tolerance rarely act in isolation. Many authors have attempted classification with little agreement and so this necessitates at least some clarification of the terminology to be used in this text.

A useful description of some mechanisms employed by microbial cells is made by Blake *et al.* 1993, who broadly grouped them into; accumulation, metabolic precipitation, and redox transformation or enzymatic detoxification. Rouch *et al.*, (1995), and Margesin & Schinner (1995) added further useful classifications which comprise metal exclusion by a permeability barrier and active pumping to exclude the toxic metal from the cell. In this review the mechanisms that bacteria employ for dealing with metals will simply be grouped by:

- passive accumulation,
- active accumulation,
- control by membrane transport systems.

Descriptions of these categories and their sub-sets are made in sections 2.3.2-2.3.4. Despite this classification it is still agreed that such grouping is largely artificial, since mechanisms to protect a cell will be employed in a cascade. Rouch and co-workers suggested that a cell's response to a toxic agent is dependent upon both cell specific factors and environmental factors (Rouch *et al.*, 1995).

Cell specific factors can include:

- whether the metal is required for growth,
- which of the cell components (cytoplasm, membrane, envelope) require protection,
- the number of uptake mechanisms that exist (i.e. whether the metal may enter easily via a range of routes).

Environmental factors include:

- availability of resistance mechanisms within the microbial population,
- the nature of the selection pressure (a gradual increase in concentration or occasional shock loads),

- the rate of change of metal levels,
- the nutrient status of the environment.

2.3.1 Control of resistance mechanisms

Resistance mechanisms although triggered by external factors are genetically controlled by information which may be:

- integrated on the chromosome,
- plasmid encoded,
- on other mobile genetic elements such as transposons or insertion sequences.

The activity of mobile genetic elements; plasmids, transposons and insertion sequences, is induced in the presence of a metal challenge and can proliferate through the microbial population as a result of the selection pressure. Genes on plasmids can provide the host with ancillary phenotypic functions not needed by the cell under all growth conditions (Silver, 1992). It is hypothesized that a tightly regulated genetic mechanism found mobile in the population may be 'cheaper' at the cellular level than maintenance of a highly specific metabolically expensive membrane protection system to allow influx of only those metal ions which are actually required by the cell (Ji & Silver, 1995).

Though the mode of transfer of bacterial resistance appears ubiquitous i.e. plasmids encoding metal resistance genes have been found throughout the eubacteria, encoding resistance to many metals e.g.: Ag^{+}, AsO_2^{-}, AsO_4^{3-}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, TeO_3^{2-} and Zn^{2+}, that is not to say that the mechanisms of the resistance are universal. Only one of the metal resistance mechanisms, that of resistance to mercury, demonstrates a high degree of plasmid homology between organisms as diverse as Gram-negative and Gram-positive (Cervantes & Silver, 1992). Resistance to other metals is rather more limited within bacterial groups. Plasmid genetic determinants for chromate resistance have been found in several genera and particularly *Pseudomonas*. Resistance to chromate is associated with decreased chromate transport by resistant cells encoded by plasmid borne ChrA genes (Cervantes & Silver, 1992)

The molecular genetics of resistance mechanisms remains largely unexplained due to a lack of background research. Experts in the field of bioremediation such as Silver, contend that the excellent opportunities for use of microbial remediation systems in industrial applications will remain under-exploited until more is understood about the biochemistry and genetics of the resistance mechanisms (Silver, 1994).

2.3.2 Passive accumulation

Metal accumulation is sub-divided by whether the process is passive or active, and further by the site of the sorption. Passive accumulation by micro-organisms can be regarded as an ion exchange process (Marquis, *et al.*, 1976). Most heavy metals can be sorbed onto the surface of microbial cells, either living or dead, through a passive metabolism independent accumulation (Gadd & Griffiths, 1978). Due to the nature of this adsorption the metal can be desorbed from the surface through a change of conditions (often pH) and the metal recovered, and the adsorbent at least to some extent regenerated (Gadd & Griffiths, 1978).

The passive sorption/complexation generally occurring at the cell envelope is often termed **biosorption** (Volesky, 1990 b). Biosorption is rapid, reversible, and occurs whether or not a source of carbon is present in the medium (Norberg & Persson, 1984). Far from being an isolated process this reversible binding may be a prelude to transport into the cell (Hughes & Poole, 1989 b), although this is not always the case (Norris *et al.*, 1976).

Passive accumulation relies upon physico-chemical mechanisms for accumulation of metal at the cell surface, or the interface of the biomass with bulk liquid, although a combination of metal binding mechanisms may actually be functional in immobilizing the metallic species on the biosorbent (Beveridge & Fyfe, 1985). The relative importance of each mechanism depends upon the external environmental factors as well as the type of metal, its ionic form, and the type of binding site responsible for sequestering the metal (Volesky, 1990 b). There are three main processes of adsorption: chemical adsorption; physical adsorption involving electrostatic interaction of Van der Waals forces (Hutchins *et al.*, 1986); and ion exchange or cation adsorption (Marquis *et al.*, 1976; Gadd, 1988: Remacle 1990 b). In reality it is

difficult to separate physical and chemical adsorption and most adsorption phenomena involve all three forms (Gadd, 1988). The passive uptake of metals by the cell envelope has been described by some authors with the same models used traditionally in the adsorption industry; the Langmuir and Freundlich models amongst others (Remacle, 1990 b; Tsezos & Volesky, 1981), although these models fail to account for the very complex nature of the cell's surface and its inherent heterogeneity.

Across the microbial genera different components of the cell envelope may be of varying importance regarding metal uptake between. The 'discrete' sites of passive metal accumulation include the capsule and the cell wall;

2.3.2.1 Cell wall

The bacterial cell wall with its nearly universal wall polymer, peptidoglycan, constitutes a macromolecular matrix which can behave amphoterically (Remacle, 1990 b). This leads to the versatility of its binding properties. Metal binding may consist of 2 stages which demonstrate that the sorption is not solely an ion exchange phenomenon (Gadd, 1988). The first step is a stoichiometric interaction between metal ions and active sites; the second, an inorganic deposition of greater than stoichiometric amounts of metal (Beveridge & Fyfe, 1985; Volesky, 1990 a).

The binding capacities of Gram-positive and Gram-negative bacterial walls differ through variations in the wall components and structure.

Gram-postive cell walls

Gram-positive cell walls consist of further layers attached to the peptidoglycan by covalent bonds. These layers, often composed of teichoic and teichuronic acids, contribute the anionic property of the wall (Remacle, 1990 b). Metal binding is mainly to the anionic sites of the cell wall (Hughes & Poole, 1989), although this will be very much organism and species specific, as is the exact composition of the cell wall.

Mohapatra *et al.* (1993) noted that the walls of *Bacillus megaterium* are composed of peptidoglycan, teichoic and techuronic acids, containing many phosphate and carboxylic groups which are responsible for metal sorption. Beveridge & Fyfe (1985)

investigated metal binding by the wall of the Gram-positive bacteria, *Bacillus subtilis*, and determined that the electronegative carboxylate groups of the peptidoglycan harboured much of the capacity. Phosphodiester groups in the teichoic acid, sugar hydroxyl groups in wall polymers and amide groups of peptide chains also contribute to metal binding (Hughes & Poole, 1989 b).

That metal binding is species and environment specific becomes clear through the same study where the walls of *Bacillus licheniformis* composed of teichuronic acids and peptidoglycan exhibited remarkably different uptake characteristics. The cell walls, containing half the peptidoglycan of those of *B. subtilis* generally accumulated less metal, and up to 20-fold less metal in the case of copper. Indeed it was found that in this species the teichuronic acids played a much greater role than did the peptidoglycan. Marquis *et al.* (1976) found that in terms of selectivity cell walls behaved much like commercial carboxylic, cation-exchange resins with a metal affinity series of $H^+ > La^{3+} > Cd^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+} > K^+ > Na^+ > Li^+$. The ion content of the cell wall was dependent upon the ionic composition of the culture medium; the metal uptake capacity reflected not only the wall composition, but also the external environmental factors.

Gram-negative cell walls

Capsulated Gram-negative bacteria are often more tolerant to metals than Gram-positive micro-organisms (Hughes & Poole, 1989 b). The capsule surrounding the cell wall, but still a part of the cell envelope, has been highlighted as a site of metabolism-independent metal accumulation (Brown & Lester, 1982).

2.3.2.2 Capsule

The cellular capsule is particularly important in the metal tolerance mechanisms of Gram-negative bacteria. Freidman & Dugan (1968) found that an extracellular polysaccharide producing *Zoogloea ramigera* accumulated twice as much metal as did a non-producing strain. Scott *et al.* (1986) demonstrated that live capsulated *Pseudomonas putida* had a greater cadmium removal efficiency at concentrations of 0.5-1.5 mg/l than did the non-capsulated *Pseudomonas cruciviae*. Cells of these pseudomonads indicated that metabolic activity played a significant role in cadmium

uptake by the non-capsulated species and also to a lesser extent in the capsulated *P. putida* (Scott *et al.*, 1986). Studies upon extracted polysaccharides have shown that the polymer will complex and concentrate metal ions (Bitton & Freihofer, 1978; Corpe, 1975; Brown & Lester, 1982; Scott & Palmer, 1988). This is due to the anionic nature of the polysaccharide components such as D-glucose, D-galactose and D-glucuronic acid which occur frequently in the extracellular capsule (Gadd, 1988; Brown & Lester, 1982; Sutherland, 1982).

2.3.3 Active accumulation

Bioaccumulation is an active uptake of metals by live cells. Intracellular accumulation is thought to occur by slow transportation through the bacterial cell wall via the extracellular ligands which initially bind the metal (Hughes & Poole, 1989 b). Gadd & Griffiths (1978) noted that the mass of metal bound by surfaces through passive accumulation was insignificant when compared to the amounts that can be taken up by energy-requiring processes. Thus although maintenance of an active cell culture presents practical difficulties in a biotechnological setting, the benefits for bioremediation applications of enhanced process efficiency through maintaining active micro-organisms are clear.

The metals accumulated and released inside the cell may be incorporated into biochemical pathways if not toxic, or trapped in an inactive form by complexation with a high affinity ligand. Metals can be altered enzymatically by transformation into a less toxic form (Ford & Mitchell, 1992).

2.3.3.1 Redox transformation

It has been demonstrated that changes of the valency of Fe, Cu and Mn may be required for them to be utilized by micro-organisms, proving that oxido-reduction systems for heavy metals do exist in these organisms (Wakatsuki, 1995). The redox reaction transforms toxic metal species to a less toxic form through the action of microbial enzymes. Reducing systems for many metals; uranium, chromium, selenium, lead, technetium, mercury, and tin are documented (Silver, 1992; Blair *et al.*, 1981; Lovely, 1995). The electron donor systems for reduction of heavy metal in the cell surface layer are found in the plasma membrane (Wakatsuki, 1995). The enzymatic

detoxification of metals to less toxic forms is best known for inorganic and organo-mercurials, oxidation of As^{3+} and reduction of Cr^{6+} (Silver, 1992). Macaskie and co-workers documented the action of *Citrobacter* cells, binding accumulated cadmium as cadmium phosphate as a method of detoxification (Macaskie *et al.*, 1987). A wide variety of micro-organisms can enzymatically reduce Cr^{6+} to Cr^{3+} and the potential of this reaction for bioremediation has been recognised (Lovely, 1995). Hexavalent chromium is dangerous to cells because of its solubility, reduction to Cr^{3+} enables cells to sequester the metal as a less soluble form, limiting its mobility and hence its damaging effect upon the cell metabolism.

The fate of metals which have been enzymatically transformed by the cell can be by compartmentalization through precipitation or nucleation, or alternatively volatilization.

Precipitation and nucleation

Precipitation and nucleation of metal is a consequence of a redox reaction within the cell which reduces its solubility. The metal becomes deposited in the microbial cell wall. Precipitation often occurs when metal concentrations are very high (Gadd & Griffiths, 1978). The amount and shape of the precipitate or nucleus is determined by the tertiary structure of the cell wall; the linkage of fibrillar and matrix components (particularly of the peptidoglycan) affects the mesh diameter and thus the degree of void space in the wall skeleton (Beveridge & Fyfe, 1985: Remacle, 1990 b). Metal precipitates may be in the form of phosphates, sulphides, carbonates, oxides and oxalates (Hoyle & Beveridge, 1984). Precipitated metal may then act as a nucleation site for secondary deposition (Hughes & Poole, 1989 b).

Volatilization

Blair *et al.*, (1981) demonstrated that Sn^{4+} and Sn^{2+} were transformed to the volatile methylated species including tetramethyl tin and hydridic methylstannanes. These volatile tin species were detected in the atmosphere above growing *Pseudomonas* cultures by GC-MS (gas chromatograph mass spectrometry), proving the system an effective means of removing the metal from the environs of the cell (Blair *et al.*,

1981). The reduction of mercury from Hg^{2+} to elemental mercury and its subsequent removal from the cell by volatilization is a well documented example of mercury resistance, the same end may also be achieved through methylation (Gadd & Griffiths, 1978). Interestingly the methylated compounds (methyl and dimethyl mercury) can be more toxic than the species originally internalized, its toxic effect within the cell however, is reduced by volatilization and release to the atmosphere.

2.3.4 Control by membrane transport systems

Regulation of intracellular concentrations of inorganic cations and anions is mediated via membrane transport systems. Under 'housekeeping conditions' abundant intracellular ions are accumulated by high rate, relatively non-specific uptake systems that are synthesized constitutively and are metabolically cheap and efficient since several cations or anions are transported by a single system. A result of this is that unwanted and even toxic metals may gain entry to the cell interior. The toxic substance constitutes a selection pressure, which can be dealt with by the mechanisms of exclusion or efflux.

2.3.4.1 Exclusion

Metal entry to the cell may be precluded by altering the membrane transport systems involved in initial cellular accumulation which are exploited by rogue metals for entry into the cell (Silver, 1992). Examples of metals that can be excluded by various metal tolerant micro-organisms include cadmium, zinc, lead, mercury, copper, cobalt and manganese. Again exclusion may be effected through a variety of mechanisms which are organism and metal specific and will not be expanded upon here but are discussed more fully by Hughes & Poole (1989 b).

2.3.4.2 Active transport or efflux mechanisms

Active transport or efflux can play a role in metal tolerance (Hughes & Poole, 1989 b; Higham *et al.*, 1984). The highly specific cation or anion efflux systems encoded by resistance genes are the most commonly found mechanisms under plasmid control (Silver, 1992). Specific examples of efflux as a metal tolerance mechanism have been found in a range of bacteria.

The metal transport of *Escherichia coli* comprises two phosphate transport systems involved in internalization of arsenate. Resistance to arsenate is therefore not due to reduced net accumulation, but through a rapid arsenate efflux driven by ATP (Hughes & Poole, 1989 b). The energy dependent Cd^{2+} resistance mechanism found in *Staphylococcus aureus* was attributable to a plasmid encoded energy dependent efflux preventing high internal cadmium accumulation (Tynecka *et al.*, 1981). A similar mechanism of more effective pumping was employed by the spontaneous mutants of *Alcaligenes eutrophus* which possessed increased resistance to nickel (Hughes & Poole, 1989 b).

2.3.5 Remediation by biomass

Some authors still contend that with advances in the understanding of microbial physiology, biochemistry and molecular genetics, bacterial cells may replace activated carbon in detoxifying metal contaminated waste-water; Rawlings & Silver, (1995) specifically targeted waste from mining concerns. Indeed some microbial biomasses have already been proved to possess a greater capacity than commercial ion exchange resins (Marquis *et al.*, 1976; Xie *et al.*, 1996).

In a recent example Sekhar *et al.* (1998) highlighted tannery effluents, paint and electroplating industries as important sources of environmental metal pollution. They used a dead fungal biomass to remove chromium, nickel, iron and calcium from synthetic solutions of up to 10 g metal per litre. Fungal uptake capacities were dependent upon solution pH. At their optimum pHs adsorption was ranked by preference, Ca > Cr^{3+} > Ni > Fe > Cr^{6+} (Sekhar *et al.*, 1998). The uptake capacity of nickel was most affected by the presence of other metal ions in solution, Cr^{6+} the least (Sekar *et al.*, 1998). Marmaril *et al.* (1997) used planktonic and immobilized *Rhizobium* cells for biosorption of Cr^{3+} and described its uptake by Langmuir and Freundlich isotherms. They achieved uptake capacities of up to 2.86 mg Cr/g dry mass for free cells and 5.0 mg Cr/g dry mass when cells were immobilized on either ceramic beads or aquacel (a porous cellulose carrier with a charged surface). Fujii and co-workers (1990) used a fed batch reactor of *Enterbacter cloacae* for reduction of hexavalent chromium but recommended that the insoluble hydroxides would then need to be removed from the reactor effluent by conventional separation technology.

The potential of *E. cloacae* for the reductive precipitation of Cr^{6+} to Cr^{3+} was also noted by Lovely (1995), although the difficulty of removing the reduced chromium from an active cell culture precludes its useful employment. Interest remains in the use of bacteria for the reduction of Cr^{6+} however since the oxidation of organic compounds can be linked through the bacterial activity to provide electrons for the reduction process (Wang & Shen, 1995).

Many other examples of the metal capacities of live and dead biomass exist, from fungi and yeast, to algae and the higher plants, and many components and products thereof. The scope of such research is too broad to list them here, yet it is clear from the literature that the engineering difficulties of reactor design and maintenance of the biomass have largely been overlooked in these treatise.

Some authors have explored the area of downstream separation processes in order to find those which may remove effectively the metal laden bacteria from the newly decontaminated waste stream at a cost which would ensure that the use of waste biomass is still economically viable. Matis *et al.* (1994) reported results for a flotation separation stage that were promising when compared with a cadmium ion flotation treatment. The biomass lacks the useful form and particle size of the existing technologies of ion exchange resins, zeolites and activated carbon, and encouraging the water industry to invest in what is likely to be expensive and specialized new technology is also unlikely to prove successful. Biosorbents must have the appropriate characteristics of hardness, porosity, particle size and density to make them suitable for process applications (Volesky, 1990 b). Modification of the biomass to form particles with the correct properties, however, can render it uncompetetive as a cheap adsorbent; *Pseudomonas aeruginosa* immobilized in polyurethane gel-beads, although effective at removing uranium from a dilute acidic waste-water (Hu & Reeves, 1997), is expensive as a technology for removal of less strategically important metals. Some processes using modified biomass such as AMT Bioclaim, a granulated non-living *Bacillus* based biosorbent (Hutchins *et al.*, 1986), and AlgaSORB a freshwater alga (Kuyucak, 1990) are commercially available.

To gain the advantageous physical properties of activated carbon leads intuitively to the development of a bacterial biofilm over a GAC support, thus removing any necessity for downstream processing, and encompassing all positive aspects.

2.4 Integrated waste-water treatment

A biofilm coated GAC, possessing the properties of both GAC and biomass, would be uniquely suited to the detoxification of metal bearing waste-waters, especially since GAC technology is already in place for the treatment of organics in waste-water. Thus a complete GAC water treatment process may be possible, which could be tailored to target problem metals by seeding columns with specific metal sorbing organisms. Conditions in the column may select for bacteria which utilize and break down organic components sorbed at the carbon surface; a limited *in situ* carbon regeneration (Chudyk & Snoeyink, 1984; Kutics & Suzuki, 1992).

2.4.1 Biological activated carbon

Granular activated carbon over which a biofilm of micro-organisms has been encouraged or allowed to develop is known as biological activated carbon (BAC). In 1979 Ying & Weber identified the potential for an integrated waste-water treatment combining biological adsorption processes with the already established technology of activated carbon. The formation of a bacterial biofilm over granular activated carbon can lead to an elongation of service time for adsorbent beds (Olmstead & Weber, 1991; Kutics & Suzuki, 1992; Voice *et al.*, 1992). Dussert & Van Stone (1994) reported that in the early nineteen seventies it was found that the bacteria which naturally proliferate on carbon columns could be implicated in the net removal of organics from carbon filters. The evidence for this phenomenon remained largely anecdotal until Chudyk & Snoeyink (1984) used bench scale GAC columns pre-saturated with phenol to discern the relative importance of biodegradation and bioregeneration.

Biodegradation is a general term for the biologically mediated breakdown, utilization or detoxification of compounds which have entered a column but remained in bulk solution, i.e. those which have not been adsorbed. Bioregeneration is specifically the

biodegradation of compounds already adsorbed with the result that they are then removed from the adsorbent. Adsorbed organics may be removed by desorption, due to a change in the localized equilibrium, through direct bacterial assimilation, or by exo-enzymatic attack (Schultz & Keinath, 1984). It was found that biodegradation of phenol circulated through a column occurred routinely, but significant carbon bioregeneration only occurred at high concentrations of dissolved oxygen (9 mg/l as opposed to 4 mg/l) (Chudyk & Snoeyink, 1984). In column tests Ying & Weber established a favourable result for reduction of the total organic carbon (TOC) content of a synthetic waste-water containing sucrose, toluene sulphonate and potassium biphthalate, using an undefined biofilm on activated carbon. The treatment of the waste by live biomass on activated carbon was superior to that of both living biofilm on non-activated carbon and metabolically inactive biomass on activated carbon (Ying & Weber, 1979).

Andrews & Tien (1981) modelled the removal of valeric acid from solution and the regeneration of the carbon by microbial activity at its surface. The authors of the paper noted that the removal of valeric acid achieved was superior to that predicted, and suggested that this was due to its effective bioregeneration. The advantages of biological activated carbon are three-fold (Andrews & Tien, 1981):

- bacteria and carbon may be complementary in the type of molecule that they remove from solution,
- the bacterial activity may not only be the cause of removal of molecules from bulk solution and near the carbon surface, but may also biodegrade molecules which desorb from the carbon, thus regenerating the carbon,
- bacterial growth may be enhanced by the presence of a carbon surface; providing protection from shock loads of toxic components by adsorption of waste products or by concentration of nutrients at its surface (Shultz & Keinath, 1984; Morsen & Rehm, 1990).

The use of an activated support for the biofilm can offer distinct advantages with respect to removal of toxic or biologically resistant compounds, and the removal of organics by biofilm may be enhanced by use of an activated support. Organisms within

the biofilm may either be protected from shock loads of components of the waste, or it may be that the compound is then available both from the bulk liquid phase and the adsorbed phase through the use of *activated* carbon. Li & DiGiano (1983) noted the advantage of activated carbon over inert supports through the enhanced growth rate of the biofilm. These authors suggested that this was due to microbial utilization of internally sorbed substrates (Li & DiGiano, 1983).

It is hypothesized that the use of an activated/adsorbent substrate and an actively metabolizing biofilm can be mutually beneficial, although some authors have noted that direct cause and effect of this nature is difficult to prove unequivocably because of the difficulty of assessing each of the processes in isolation when they are in fact interdependent (Shultz & Keinath, 1984).

Researchers have noted the advantage of an activated carbon/biofilm to overcome toxicity problems. Wang & Shen (1995) discovered that bacterial reduction of Cr^{6+} was inhibited and cell growth compromised in the presence of phenol, a frequent co-contaminant in chromium containing waste. An activated carbon substrate can adsorb the shock loads of phenol, sequestering it from organisms, thus facilitating cell proliferation and metal detoxification in what would otherwise be extremely unfavourable conditions for microbial activity.

Kuticks & Suzuki (1992) found that attachment and growth of a bacterial biomass on the surface of activated carbon was related to the surface characteristics, notably enhanced growth came with increasing surface acidity. Kida and co-workers implicated a number of factors in the attachment of bacteria to inert and adsorbent granular support media. These authors concluded that an enhanced surface area was immaterial, and that a certain degree of roughness and a positive surface charge were the most important parameters driving bacterial adhesion (Kida *et al.*, 1990). Dussert & Van Stone (1994) reported that the biological removal of dissolved organics with activated carbons from the sources, coal, lignite and wood, was independent of the raw material.

2.4.1.1 Industrial applications of BAC

Biological activated carbon is a specific term for the use of granular activated carbon as a support for growth of a microbial biofilm, although other commercial systems use the merits of a carbon adsorbent and microbial biomass in a subtly different way. The Powdered Activated Carbon Treatment (PACT) process involves addition of powdered activated carbon (PAC) to activated sludge biological treatment systems providing enhanced process stability. An addition of 100 mg/l PAC aided removal of all target compounds and demonstrated a synergistic effect by removal efficiencies beyond what might be achieved by the processes in isolation (Olmstead & Weber, 1991).

Some disadvantages of microbial growth on activated carbon have been highlighted but these are problematic only for certain specific applications. LeChevallier *et al.* (1984) noted that pathogens attached to GAC may escape disinfection by chlorine. Attached cells are less susceptible to biocides than their planktonic counterparts (Menezes *et al.*, 1995), which may in part be due to adsorption by the carbon or through development of an extracellular polysaccharide layer by the micro-organisms. This phenomenon is mainly of concern when activated carbon is used in the treatment of potable water and presents only a small risk when treating wastes such as those from dump-sites. Interference in the efficacy of carbon sorption by the presence of biofilm has been postulated at two levels; firstly that film covering the carbon granules can affect mass transfer, leading to a slower attainment of equilibrium (Shultz & Keinath, 1984), and secondly that some cell products may be adsorbed by the carbon onto sites which would otherwise be occupied by target molecules, indeed a competition for active sites (Rittmann & Randtke, 1980; Olmstead & Weber, 1991). The latter is only a significant problem when the criterion for regeneration of a column is breakthrough of a non-biodegradable target material. In contrast De Laat and co-workers suggests that the biodegradation of some components of the mixed waste, by a biofilm on F400 lead to an increase in the adsorption capacity of the carbon for non-biodegradable solutes, although the kinetics of the uptake were not studied (De Laat *et al.*, 1985).

Results and recommendations are very much dependent upon experimental design since Koch & co-workers (much against the tide of opinion) reported that a sand/biofilm system was superior to GAC/biofilm system for the degradation of phenol. This was related to a slower but more extensive development of biofilm over the sand (Koch *et al.*, 1991).

2.4.2 Metal sorption by biological activated carbon

Mohapatra and co-workers (1993) noted the positive effect of a *Bacillus megaterium* biofilm upon copper accumulation by activated carbon. Their claims of synergy were however inaccurate since the uptake achieved by the combined system was not greater than the sum of the accumulation by biofilm and activated carbon in isolation. Scott *et al.* (1995) further demonstrated the potential of biological activated carbon through the enhanced removal of Cd, Cu, Ni and Zn from single component metal salt solutions.

2.4.3 The scope of current work

There is much anecdotal evidence within the waste-water industry to suggest the great potential for use of an activated carbon support covered with a metabolizing biofilm for the remediation of waste-waters containing a cocktail of toxic components when neither component in isolation could effect a total decontamination. There is a growing body of literature on the adsorption of metals from solution in addition to the traditional uses of activated carbon for adsorption of organic compounds such as aromatics, phenol, pesticides and dyes.

Little comprehensive research is available however, upon the capacity of an activated carbon system for toxic metals Cr, Cd and Ni, which has assessed the qualifying effect of the biofilm and growth media. The current study has undertaken to assess the suitability of carbon for biofilm development and to quantify the uptake capacity of the carbons for the three metals and metal mixtures, with and without biofilm using a laboratory model of *Klebsiella pneumoniae* covered activated carbon.

Materials & Methods

The chapter has been divided into two sections. In Section 3.1 entitled 'Method development' an explanation is made of the choice of carbons, metals and micro-organisms used within the experimental work. The methods that are available for metal determinations and biofilm quantification are discussed and the relative merits of

the techniques are discussed with reference to the requirements of the present study. The experimental methods selected following this review are described fully in Section 3.2 'Experimental materials and methods', where the experimental materials and protocols are detailed.

3.1 Method development

The reported study is to investigate the conditions under which a carbon/biofilm system would be practicable for metal accumulation without compromising the inherent adsorbing capacity of the granular activated carbon (GAC) for impurities. Comparison of virgin carbon with the biofilm system can be made by use of adsorption isotherms which show the distribution, at a constant temperature, of impurities between the adsorbed phase and bulk solution at equilibrium. The isotherm plot presents the amount of impurity adsorbed per unit weight of carbon versus that remaining in solution. Isotherms of metal uptake by carbon and carbon/biofilm systems have been produced to assess the feasability of each system for metal uptake applications. Data was gained from batch equilibrium experiments in accordance with the carbon industry standard for determination of the adsorptive capacity of activated carbon by the aqueous phase isotherm technique (ASTM, 1989).

3.1.1 Granular carbons

Carbons representing a broad spectrum in liquid phase applications were available. Having examined the specifications of these carbons (such as source material, application, total surface area, iodine number and methylene blue number) it was considered that the most valuable and wide ranging information could be gained by using carbons with gross differences. Granular adsorbents (rather than powdered carbons) were chosen for their suitability for column operation in potable water applications.

Activated carbons; Filtrasorb 400 (F400) and Picabiol, were compared with a calcined carbon; anthracite. Filtrasorb 400, a bituminous coal based activated carbon with an extended surface area, is widely used in potable water treatment for removal of

organics including pesticides, micropollutants, humic acid and detergents. Anthracite has a relatively low surface area and represents F400 in the pre-activated state. Picabiol, a lignite based carbon, is promoted as a specialist carbon for biofilm development. Carbons were wetted before use to ensure that the entire carbon surface was immediately available for transport of impurities from the bulk solution to internal carbon surface by diffusion.

3.1.2 Metals and metal measurement

Poor controls and insufficient legislation in the past have allowed industrial metal wastes to be discharged into water courses. Although the metal concentration in the waste may be low natural biotia can effect its accumulation to dangerous levels. Early pollution prevention efforts amounted to containerization of waste before dumping, but corrosion of the vessels has since led to seepage of the toxic contents into the soil to be leached into the water table by rainfall. Thus initial attempts at prophylaxis have themselves established waters containing low or trace concentrations of mixed toxic metals.

Three metals, chromium, cadmium and nickel, are highlighted as important for study because of their prevalence in industrial waste-waters, and therefore their expected presence as pollutants in natural bodies of water and dumps. The metal valence state and species selected for use were those likely to be the major component of waste from industrial activity and, in the case of chromium, the valence which is most toxic:

- Chromium, in the form of chromium trioxide (CrO_3), used in chromium plating, tanning of hide, paints and as a textile mordant. Hexavalent chromium (Cr^{6+}) causes irritation to and corrosion of tissue. Elemental and trivalent chromium are relativley non-toxic;
- Cadmium, in the form of cadmium chloride ($CdCl_2$), is a component of electroplating baths;
- Nickel, as nickel chloride ($NiCl_2$), used in electroplated nickel coating.

Atomic absorption spectrometry (AAS) is routinely used in the water industry to measure heavy metals in waste-water. It is sensitive, specific, accurate, economical, rapid, and can determine a broad range of elements; however analysis of specific

metals can be subject to interference (Sterritt & Lester, 1980). This interference is not only due to other metals (for example, absorption of chromium is suppressed in the presence of iron), but also some organic and inorganic compounds (e.g. phosphate) which can be present in water samples.

Gallorini *et al.* (1993) noted that sample components such as chlorides, sulphides, bromides and organic matter, reduced the sensitivity and precision of trace metal analysis. They measured metals by AAS with a graphite furnace finding that Cd, Cu, Co, and Pb were affected most by interference. At 1 µg/l cadmium suffered 25% signal suppression in the presence of 0.1% Cl^-, 20-25% suppression in 0.1% S^{2-} and 100% suppression in a matrix containing organic matter with a COD (chemical oxygen demand) of 5 g/l. Copper at 20 µg/l suffered 5-10% suppression in Cl^- but 10% enhancement in S^{2-}. Other analytical equipment was tested and also found to be affected by interference; neutron activation analysis, one of the most sensitive techniques for many trace metals, was sensitive to sodium and bromine at 0.5-1%, preventing quantitative analysis of many trace elements (Gallorini *et al.*, 1993). In this study components of the microbial growth media particularly, were identified as possible causes of interference.

Sterritt & Lester (1980) described pre-treatments for environmental samples to improve the accuracy of analysis. Sludges, which must be tested for metal content before land application (Cottenie, 1981), undergo a rigorous procedure to remove the high concentration of solids, either by digestion with strong acids or by dry ashing in a furnace (Doyle *et al.*, 1980). These pre-treatments are inherently lengthy and end points can be subjective, reducing the suitability of the technique for routine and frequent analysis. Unsuitable extraction methods may also result in poor recoveries. Some acid extractions are specific to metal and matrix and inappropriate use can lead to formation of insoluble species, or volatile compounds lost at later stages of the processing (Sterritt & Lester, 1980). Similar treatments to remove nutrients from the growth medium can be precluded from this study for reasons of sample paucity and, especially in kinetic studies, the need for immediate analysis.

Gallorini *et al.* (1993) concluded that of seventeen different metal determinations in leachate samples by AAS, fourteen required lengthy chemical separations, such as digestion and Chelex column separation. Attempts to shorten the procedure by use of microwave dissolution resulted in poor recoveries and only Cr, Mn, Ni, and Zn could be directly determined by AAS.

A review of the literature on analysis methods and pre-treatments made it clear that no single method was available for the three test metals. To maintain comparable experimental procedures it was decided that quantification of the interference effects by growth media, and other components of the system, followed by correction for that interference if necessary, would provide the most accurate analysis.

The difficulty of preserving chromium samples was discussed by de Beer & Coetzee (1988). They sustained losses of 80% per week under storage conditions, which was reduced to a 20% loss when buffered. In the light of this, experimental samples were left unbuffered, to reduce interference, but were analysed without delay and within 1 hour of collection.

3.1.3 Micro-organisms and media

The micro-organisms chosen for this study were Gram-negative bacteria noted for their production of a slime capsule, or glycocalyx, composed of extracellular polysaccharides. Previous studies (Palmer, 1988; Karanjkar, 1994) demonstrated the potential of such organisms for metal uptake and for growth in integrated carbon/biofilm adsorption systems. Exopolysaccharide (EPS) producing cells are well suited to biofilm production since the excreted matrix aids cell attachment (Allison, 1994). It may be considered that a growth medium designed for maximum EPS production is optimum for biofilm development over the granular activated carbon. Sessile organisms (in a biofilm) have an altered level of activity compared with planktonic cells, low nutrient levels in solution may favour a biofilm existence (Videla & Gaylarde, 1992) and it is starved cells that are thought to produce more exopolysaccharide (Fletcher, 1987). A minimal medium may improve the quality of biofilm, but increase generation times adversely, and so a compromise medium was sought. The required nutrient source should not interfere with the analytical

equipment (Section 3.2.5) nor saturate the carbon preventing metal uptake, but would allow proliferation of cells and production of EPS in as short a time span as possible. A range of experiments was undertaken to identify such a medium.

Ringer's solution, a salts medium isotonic with micro-organisms, would also be a useful adjunct to the metal test solutions as an aid to cell homeostasis. This would help to ensure that the biofilm remained intact on the carbon surface when challenged with the metal solution and during the period of equilibration following. Thus Ringer's solution was tested for its suitability regarding its effect upon metal analysis and carbon performance.

3.1.4 Quantification of biofilm formation on carbon

Valid conclusions regarding the efficacy of different carbons for biofilm development, and the adsorbing properties thereof, may only be drawn if an attempt has been made to assess the microbial growth achieved on each carbon sample. The quantification of biofilms has long posed a problem and the importance of which parameter is to be measured (i.e. the number of cells held within the biofilm, the exopolysaccharide excreted by the cells, or the total biomass) is determined by where the biofilm is found, and the industry perspective upon the ecology and significance of the growth, for example:

- In the food industry it could be the number of food poisoning organisms within a biofilm on food preparation equipment that is of greatest importance. In this example a *live cell count* would hope to establish if an infective dose had accumulated;
- The efficiency of industrial scale equipment is affected by the biofilm load on its surface. Measurement of the *total biomass* including cells and exopolysaccharides, may indicate the loss of efficiency due to the fouling;
- In aspects of bioremediation both metabolic uptake by cells and passive uptake by excreted exopolysaccharides can be important for removal of constituents from a solution, thus a measure of both *cell number* and *exopolysaccharide mass* may provide the best reflection of useful biomass within a bioreactor. The procedures for such a measurement however are lengthy and comprise many

steps which can introduce inaccuracies, furthermore large sample masses are required to negate the effects losses. A large sample mass is unavailable due to the small scale of the batch equilibrium tests in the current study.

The many methods of biofilm measurement may be broadly categorized (Characklis, 1990):

- direct measurement; of biofilm quantity,
- indirect measurement; of transport properties through the film,
  of microbial activity,
  of specific biofilm constituents.

3.1.4.1 Direct measurement

Direct measurement involves measuring biofilm thickness or mass, no differentiation of the component parts is made, and the biofilm is regarded as a whole. Assessment of thickness may be made by means of optical microscopy; focusing first upon the biofilm surface then the substratum below. The height difference is measured by calibrations on the microscope and the thickness calculated using the refractive index of the film. Measurement of thickness requires the biofilm to be visible with a light microscope, however visualization of the biofilm is likely to be hampered as carbon will obscure the light pathway. Calculation of total biofilm requires an estimate of the surface area of carbon available for colonization and also would rely upon a uniform or homogenous layer of constant thickness; uniformity is not a characteristic of biofilms. Direct measurements of mass, by methods such as dry weight, lack the sensitivity required for routine biofilm quantification of the batch studies described later.

Scanning electron microscopy enables direct visualization and suitable sample preparation will fix both cells and exopolysaccharides. This technique provides useful information upon colonization and biofilm structure but is predominantly qualitative. Safferman *et al.* (1993) devised a systematic scanning electron microscopy technique for evaluating biofilm coverage of granular activated carbon. Although reported as effective, the procedure was considered too time consuming and expensive for routine comparisons.

3.1.4.2 Indirect measurement

Indirect quantification by use of transport properties through the biofilm, such as friction or heat transfer resistance, is limited in its application by the necessity for technology which can only be employed in specifically designed reactors (Characklis, 1990).

Indirect measurement may also be made through microbial activity within the biofilm, using for example:

- viable cell count,
- epifluorescence microscopy,
- adenosine tri-phosphate (ATP) quantification,
- substrate removal rate.

Measurements of this kind are best suited to actively growing populations, or those with a predominance of live cells. Viable cell counts must rely upon the assumption that all cells have been removed successfully from the carbon surface and not disrupted by the mode of removal. Sonication is the most effective method of removing cells, but is harsh and likely to cause lysis and therefore an erroneously low estimate of the biofilm by viable cell count. Analysis of ATP following cell disruption is fast, sensitive and simple, but detects only viable cells (Characklis, 1990) and requires equipment and reagents that make the technique expensive. Epifluorescence, although employed successfully to count cells upon steel coupons (Menezes *et al.*, 1995), once again is subject to difficulties through the granular and opaque nature of the carbon. The use of activated carbon presents further complications since the substrate removal rate by cells can only be determined when their substratum is non-adsorbent. Activated carbon, however, is an adsorbent support which may also be instrumental in substrate removal, so producing an erroneously high estimate of the removal rate. Other parameters such as impedance, capacitance and conductance offer alternative means of indirect growth measurement, but these require expensive equipment which was unavailable.

Specific biofilm constituents can offer some of the simplest means of measurement. Total organic carbon (TOC) and polysaccharides can be measured by chemical assay, however sensitivity is limited. Alternative methods require expensive analytical equipment and lengthy preparation procedures.

The average protein content of bacterial cells is approximately 10 % (Read, 1995). This can be satisfactorily and cheaply measured by protein assay, which is of sufficient sensitivity to detect 10^5 cells. Removal of the biofilm by sonication is then advantageous since cells must be disrupted to release the contents. The technique requires no sophisticated equipment, reagents are inexpensive and readily available, and the assay is reproducible. This protein determination method was employed for routine assessment of biofilm on the carbon surface.

3.1.5 Carbon dosage of batch adsorption studies

Carbon suppliers recommend that for treatment of waste-water with an organic content of < 100 mg/l, a contact time of 45-90 minutes would be required, and a carbon dosage of between 0.2 and 5.0 kg per m^3 waste-water. In the course of these experiments a batch flask mode was operated, using 0.5 g carbon and 200 ml of 'waste-water', which is equivalent to 2.5 kg carbon/m^3, i.e. in the upper region of the recommended dose. It was found during the course of experiments that such a dosage when combined with the range of metal concentrations chosen achieved an uptake equilibrium between adsorbed metal and that remaining in the bulk. The metal left in solution was at such a level that the uptake was significant whilst the concentration remaining in bulk solution was still measurable.

3.2 Experimental materials and methods

3.2.1 Carbon

Three carbons were compared: F400; anthracite, a non-activated carbon of the same source material (Chemviron Carbon Ltd., Oldham, U.K.); and Picabiol (Wright & Company Ltd., NSW, Australia). All carbons were wetted in an appropriate medium before use. This involved autoclaving (15 minutes at 121°C) to ensure sterility,

followed by incubation for 24 hours at 25°C, shaking at 170 rpm (Controlled Environment Incubator Shaker, New Brunswick Scientific Co. Inc., U.S.A.).

Surface characteristics of the carbons were examined by use of scanning electron microscopy (SEM) (Section 3.2.12). Preparation involved mounting of the samples and sputter coating with gold to ensure conductance.

3.2.2 Carbon porosity measurements

Studies of the porosity of the carbons, to compliment the qualitative information gained by SEM, were made using a Mercury Porosimeter (Autopore 9220, Micromeritics Ltd., Bedfordshire, U.K.). The fraction of carbon available for microbial colonization may be estimated from the distribution of pore sizes. That fraction of the surface area held within pores of less than 1-2 μm in diameter would be largely inaccessible to the bacterial cells used in this study (Section 3.2.3).

The sample vessel was loaded with an accurately measured mass of carbon. This amounted to approximately 0.3 g of the activated carbons and 1.2 g of non-activated carbon, due to its greater density. All gas was evacuated from the sample vessel and replaced with mercury whilst under vacuum. Pressure applied to the mercury forced it first into the interparticle voids, and as the pressure was increased, into the pores within the carbon. The vessel's stem volume was 0.3920 ml, and the pressure with which mercury was forced into the pores was varied between 0.47 and 60,000 psia. At a contact angle of 130°, the upper pressure limit corresponds to a mean pore diameter of 0.003 μm (using a standard pressure table). Pressures greater than 60,000 psia lead to questionable interpretation of the data regarding the nature of contact angles and surface tension in pores not much wider than the diameter of a few mercury atoms. Intrusion and extrusion curves for each of the carbons were obtained in this way. An incremental intrusion plot was used to depict the degree of porosity of the carbons within the range of pore diameters 0.01-50 μm.

3.2.3 Organisms and growth conditions

The organisms under investigation were Gram-negative bacteria chosen for an ability to produce slime capsules composed of extracellular polysaccharides. Organisms were

obtained from the National Collection of Industrial and Marine Bacteria (Aberdeen, U.K.) or isolated from an industrial carbon column:

- *Klebsiella pneumoniae* subsp. *pneumoniae* (NCIMB 8806);
- *Enterobacter aerogenes* (NCIMB 88);
- *Zoogloea ramigera* (NCIMB 11941), an accumulator of heavy metal ions isolated from a trickling filter;
- *Pseudomonas elodea* (NCIMB 11942);
- *Agrobacterium radiobacter* (NCIMB 8149);
- Gram-negative isolate from a waste-water column.

Organisms were maintained at 4°C on nutrient agar (Code CM3, Unipath Ltd., Hampshire, U.K.) and subcultured regularly from stocks kept in liquid nitrogen. Starter cultures were inoculated into 5 g/l tryptone (Section 3.2.6) (Code L42, Unipath Ltd., Hampshire, U.K.) and incubated at 25°C, a compromise between an optimum for growth and realistic ambient temperature for an externally situated column.

3.2.4 Metal solutions

Stock solutions of 5 g/l metal were used to make all concentrations required for metal uptake studies. The metal compounds used: chromium (VI) oxide (Fisons, Loughborough, U.K.), nickel (II) chloride and cadmium (II) chloride (Merck Ltd., Leicestershire, U.K.); were of analytical grade. Distilled water (d.H_2O) was used to make solutions and to rinse glassware.

Analysis of distilled water by inductively coupled plasma (ICP), to ensure that contaminating metals were not present, was done by Chemviron Carbon Ltd., Research Department, Grays, Essex. Levels of the test metals were generally 3 orders of magnitude below that used in experimentation and therefore below the sensitivity of the atomic absorption spectrometer.

Glassware was treated to prevent the adsorption of the test metals to surface of the glass. Treatment comprised leaching with concentrated nitric acid (HNO_3) to draw any pre-adsorbed metals into solution as the metal nitrate and thorough rinsing

followed, when dry, by dimethyldichlorosilane in 2% 1,1,1-trichloroethane (Merck Ltd., Leicestershire, U.K.) to endow water repellance (Palmer, 1988).

3.2.5 Metal measurement by atomic absorption spectrometry

Metal concentrations were measured by flame atomic absorption spectrometry (AAS) (Perkin Elmer AAS 3110) using acetylene fuel. Conditions were optimized using a non-absorbing copper flame at wavelength (λ) 324.8 nm. Three repetitions were made of each sample with an integration time of 2 seconds, a suitable compromise between accuracy and minimization of the sample volume required. Metals were measured at the following wavelengths: chromium, 357.9 nm; nickel, 231.1 nm; and cadmium, 228.8 nm.

Atomic absorption standards, supplied by Sigma-Aldrich Co. Ltd. (Dorset, U.K.), were used for calibration of the AAS.

3.2.6 Assessment of interference effects

3.2.6.1 By potential growth media

The AAS flame was made orange by the presence of nutrient broth (Code CM1) indicative of possible interference, hence the following growth media were investigated to find if the effect could be reduced:

- tryptone (Code L42);
- diammonium hydrogen citrate $(NH_4)_2.HC_6H_5O_7$ (Merck Ltd., Leicestershire, U.K.);
- peptone (Code L37);
- yeast extract (Code L21).

All microbiological media were supplied by Unipath Ltd. (Hampshire, U.K.) unless otherwise stated (Appendix 1). Sterile media, at concentrations of 10 g/l and 5 g/l, were dispensed into 10 ml aliquots and the appropriate volume of 5 g/l stock metal solution was added to produce solutions between 5-25 mg/l for Cr(VI) and Ni(II), and 1-5 mg/l Cd(II) (the upper concentration limit was dictated by the calibration range achievable with AAS). The AAS readings were compared with those gained by

adding the same volume of metal solution to controls of 10 ml of sterile distilled water. Triplicate aliquots provided averages and reduced errors. Dilutions of the test media were also investigated as higher metal concentrations would necessarily be diluted.

3.2.6.2 By dilute growth broths and carbon leachates

Leachates from the carbon were also tested. Carbons (0.5g) were wetted with 200 ml distilled water, autoclaved and then shaken at 25°C and 170 rpm for 3 days before the solution was discarded, the carbon rinsed briefly and a further 200 ml sterile distilled water added and incubated for 7 days, in emulation of the usual test routine (described Section 3.2.8). This water, containing any leachate still to be drawn from the carbon following the wetting process, was filtered and used to dilute concentrated metal solutions to between 5-35 mg/l before measuring as before. Leachates of tryptone wetted carbon were assessed likewise to provide a realistic estimate of the interference implicit in the system.

3.2.6.3 By buffer

The interference by media used routinely for the control of experimental conditions was assessed likewise. Buffers in the physiological pH range (i.e. pH 7.2) were tested; phosphate buffer and tris/HCl, as was ¼ strength Ringer's solution (Lab M, Lancashire, U.K.) (see Appendix 1).

3.2.7 Metal uptake by planktonic cells in single metal solution

The potential displayed by each of the organisms for concentration of heavy metals from solution was first examined using cells in suspension, i.e. planktonic cells growing in a culture medium. The resilience of the organisms was tested by inoculation into 5 g/l tryptone growth medium containing between 5-100 mg/l of added test metal followed by incubation at 170 rpm and 25°C, no analysis was made of the quantity of free metal ion in solution. Triplicate flasks were examined daily for turbidity over a period of 8 days.

The accumulation of metal by cell suspensions was tested also in triplicate. Media (100 ml) was inoculated with 100 µl of the test organism and incubated for 7 days at

170 rpm and 25°C before sterile 5 g/l stock metal solution was added to give a final concentration of 20 mg/l. Following a further 3 days incubation 10 ml samples were removed, centrifuged, and the supernatant filtered with 0.45 and then 0.1 μm cellulose nitrate filters (Whatman International Ltd., Maidstone, U.K.) before analysis. The concentration of metal in solution was compared with that of uninoculated control flasks. Filters were tested to ensure that they did not adsorb metals by repeatedly passing a solution of known metal concentration through a series of the filters. No reduction in the metal concentration was detected following repeated filtration.

3.2.8 Metal adsorption isotherms

Adsorption experiments were done in triplicate and in accordance with ASTM activated carbon standard for determination of adsorptive capacity (ASTM, 1989). Comparisons were made between the metal loadings of: biofilm covered carbon; carbon in sterile growth medium; and carbon in sterile distilled water. The carbon in sterile growth medium was a control to ensure that any change in loading could be attributed to the organisms. Dried carbon (0.5 g) was weighed into 500 ml Erlenmeyer flasks and 200 ml of either distilled water, or 5 g/l tryptone medium added. The flasks were then sterilized by autoclaving. This also served to wet the carbon surface by expelling any air from within the micropores.

Biofilm was established by inoculation of triplicate growth medium filled flasks with 100 μl of an 18 hour *K. pneumoniae* culture followed by incubation for 7 days at 25°C with shaking at 170 rpm. Control flasks containing water or tryptone were incubated similarly. Following incubation the liquids were decanted aseptically and the carbon rinsed in 5 ml sterile distilled water to remove unattached cells before 200 ml of sterile metal solution at a range of concentrations (5, 10, 20, 30, 50, 100 mg/l) was added. In the study of mixed metal systems ternary metal solutions were prepared; these contained each of the metals; Cd(II), Ni(II) and Cr(VI), at two concentrations, 20 and 50 mg/l. Flasks were then returned to the incubator at 25°C for 3 days until metal uptake equilibrium was reached. Three days was established as sufficient time for equilibrium to be reached through a time course experiment (Section 3.2.9).

Samples (5 ml) were removed from each of the triplicate flasks and filter sterilized to remove cells, cell debris or carbon fines which interfere with analysis by AAS. Samples were analysed without delay and usually within one hour of collection. The concentration of metal remaining in solution was measured and compared with the initial concentration. Metal loading was calculated by mass balance. The equilibrium pH values of metal solutions in the flasks were noted.

3.2.9 Establishing a batch adsorption equilibrium

A time course experiment was undertaken to ascertain a suitable incubation period for equilibrium to be established. Two Erlenmeyer flasks, one containing 0.5g dried F400 carbon, were wetted with 200 ml of accurately measured distilled water. Into each flask 5 ml of a concentrated Cr(VI) stock solution was added accurately by pipette to achieve a concentration of 20 mg/l. The second flask, not containing carbon was a control to provide an accurate measure of the starting concentration, since adsorption would occur immediately upon addition of metal to the carbon system changing the metal concentration before a sample could be taken and measured. Flasks were incubated under the usual experimental conditions with shaking at 25°C. In the early stages of the time course experiment samples (2 ml) were taken frequently, but as the rate of adsorption was seen to slow the sample intervals lengthened until samples were taken every 24 hours. Care was taken not to remove more than 10 samples of 2 ml from the reaction vessel so as not to erode significantly the amount of metal available for adsorption.

3.2.10 The effect of spent media upon metal adsorption by carbon

The effect of a growth medium upon metal adsorption by carbon was investigated by thorough controls. It was important to ensure that the metal uptake noted when organisms were present on the carbon surface was due not simply to changes in the media components effected by the organisms. After 7 days of growth, the spent tryptone medium was harvested by centifugation at 2,600 **g** for 30 minutes (Sorvall RC-5B, Du Pont Instruments, Hertfordshire, U.K.), followed by filtration to remove any remaining cells. Aliquots (200 ml) of the harvested spent media were added to 0.5 g carbon and controls of 200 ml fresh medium and fresh medium to be inoculated for

biofilm development were made. Flasks were set up in triplicate and all were autoclaved, biofilm flasks were then inoculated. The flasks were incubated with shaking for 7 days and then broths replaced with sterile metal solution and allowed to equilibrate for 3 days before analysis.

3.2.11 Biofilm quantification

An assessment of biofilm attached to the carbon surface was made by measuring the protein recovered from cells using the BioRad Assay (BioRad Laboratories, Hertfordshire, U.K.). In this way it was ensured that comparable biofilms were achieved in the triplicate isotherm flasks, any flasks where protein recovery from the carbon was poor were discounted from the sample since it was assumed that the biofilm might not have developed on the carbon. The protein assay made it possible to estimate how effectively the organisms had colonized the three different carbon types used as supports.

The metal solution was decanted aseptically from biofilm covered carbon. The carbon and flask were then rinsed twice with 5 ml aliquots of sterile distilled water (SDW) to remove any unattached cells. A further 5 ml SDW was added and the liquid and carbon pipetted into a plastic test tube on ice. Samples were sonicated on ice, with a probe ultrasonic disintegrator (MSE 150 watt, Sussex, U.K.) for two 2 minute periods at 10 μm peak amplitude, with an interval of 4 minutes resting on ice. A time course study established this regime as providing optimum conditions for protein release. Subsequent steps were performed without delay to minimize protein breakdown by proteases released by the disruption. The sonicated solution was filtered (0.45 and 0.1 μm filters) to remove carbon fines known to interfere with the assay and a 0.2 ml sample added to 0.8 ml of BioRad assay reagent for the microassay. This was compared with a bovine serum albumin (BSA) standard curve.

Biofilms incubated for 1, 2, 3, 5, 7, 10 and 15 days were disrupted in this way and the sonicate assayed to find the optimum culture time i.e. when the maximum biofilm was present on the carbon surface.

Quantification by BioRad assay was compared to a qualitative appraisal made by electron microscopy.

3.2.12 Electron microscopy of biofilm samples

A JEOL 6310 scanning electron microscope (Japan Electron Optics Ltd., Japan) was used to study biofilm coverage of the carbon. Different preparation procedures were used to ascertain which would be best suited to visualizing the biofilm in a life-like state.

Virgin carbon samples were mounted on planchets and sputter coated with gold. In the case of biofilm coated carbon, the culture broth was poured off and the carbon rinsed with ¼ strength Ringer's solution. Samples were removed carefully from the flask using a plastic Pasteur pipette and suspended under dampened tissue paper in a sterile Petri dish until prepared and mounted.

3.2.12.1 Freeze drying

Four carbon granules were taken at random, and with minimal manipulation to avoid damage, were freeze dried, mounted on a planchet and sputter coated with gold.

3.2.12.2 Chemical fixing

A double fixation technique was used. A pre-fix in 1.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) with 0.1% $MgCl_2$ and 0.05% $CaCl_2$, for 60 minutes at room temperature was followed by 15 minutes of washing in the same buffer. An 80 minute post-fix in 1% osmium tetroxide in cacodylate buffer preceded a wash in distilled water and then a dehydration step. This involved a series of actetone baths of increasing concentration; 30, 50, 70, 80, 90, 95, and 100% (vol/vol aq) for 10 minutes each, and two baths of 100% dry acetone. Samples were critical point dried, mounted and gold coated.

3.2.12.3 Cold stage fixing

A JEOL 6310 electron microscope with cryogenic unit was used. Samples were mounted and pre-cooled in liquid nitrogen before transfer, via an air lock preparation chamber under vacuum, to the specimen chamber. This was maintained at -180°C by liquid nitrogen to ensure that hydrated specimens did not gradually freeze dry in the vacuum of the microscope, a phenomenon which can occur at temperatures above -130°C. Water was sublimed from the sample surface by raising the specimen temperature to -80°C briefly before returning the stage to the -180°C working temperature. This enabled the water film and ice crystals, which can obscure the surface detail of the micro-organisms, to be removed. Samples were sputter coated with gold in the preparation chamber.

3.2.13 Accuracy and reproducibility

All experiments were done at least in triplicate. The raw data was averaged and the average values plotted on the isotherm graphs. Standard deviations were calculated for the adsorption experiments although they were not presented graphically since the variations would be insignificant on the logarithmic scale. All data was within a limit of 2 standard deviations of the mean.

Stock metal solutions were made using volumetric glassware (grade A) of accuracy better than ± 0.1% and a balance correct to ± 0.1 mg. Larger volumes of dilute metal solutions (i.e. 200 ml) were measured to an accuracy of ± 0.01 ml. Carbon was weighed accurately to 0.5000 ± 0.0002 g and the dry media components to 5.00 ± 0.01 g/l.

Method Validation

4.1 Quantification of interference effects

A number of aspects of the experimental protocol were highlighted in Chapter 3 for clarification regarding media effects upon: a) analysis of the experimental metal samples (see Section 4.1.1) and; b) the validity of the metal removal from bulk solution attributed to the biofilm (Section 4.1.2). A series of experiments was undertaken to assess the sensitivity and accuracy of metal analysis under the experimental conditions, and another to determine the carbon and biofilm's capacity for metal, distinguishing it from uptake variations due only to altered conditions within the reaction vessel. The results of these investigations are presented in Section 4.1 in validation of the protocol used to generate the isotherm data of Section 5.4.

4.1.1 Assessment of interference upon metal analysis

There were three additions to the reaction vessel which had an unknown effect upon metal determinations by atomic absorption spectrometry (AAS), these were: the culture medium, added to the shake flask to enhance the biofilm development; the leachate from the surface of activated carbon, a natural result of wetting the carbon; and; the buffers which could be added to stabilize the conditions within and between the duplicate reaction vessels.

4.1.1.1 By growth media

It has been noted both by researchers and equipment manufacturers that metal analysis by atomic absorption spectrometry can be subject to interference by components of test solutions (e.g. phosphates, chlorides, sulphides) and high levels of organic matter (Sterrit & Lester, 1980; Gallorini *et al.*, 1993; Anon., 1982). The bacterial growth media necessary for culture of cells can be complex and may be poorly defined (See Appendix 1) possibly containing some of these components in sufficient quantities as to cause interference with the AAS analysis. Some of the culture media investigated (for use to feed the biofilm) in these experiments contain digests of proteinaceous material such as peptone, which are indeterminate and may affect the analysis. Pre-treatments to remove interfering components are involved and generally require large sample volumes (Anon., 1982), thus it was hoped to find a growth medium which had either no effect upon the analysis, or a minimal effect which could be quantified and described by an equation; data then being corrected or normalized if necessary.

Nutrient broth (NB), a non-specialized culture medium produced an orange, roaring, flame when aspirated. Since the flame conditions required for maximum sensitivity in cadmium and nickel determinations were lean, blue and oxidizing it was thought that this orange flame possibly caused by an excess of sodium ions, could compromise the AAS sensitivity. Chromium although requiring a fuel rich (reducing) flame may also be affected by these conditions since a *slightly* luminous flame is recommended for detection.

The effect of each potential growth medium upon metal detection was monitored. Components of the nutrient broth (i.e. peptone, yeast extract) were also tested. These media (in contrast to NB) contained no added salt and were less rich in nutrients creating conditions which reflect more closely those within an industrial biofilm-coated carbon column. A sample of the data is reproduced in Figure 4.1 below: the effects of two media; nutrient broth (10 g/l) and tryptone (5 g/l) upon nickel and cadmium measurement at concentrations ranging from 5-25 mg/l and 1-6 mg/l respectively are presented. The narrow concentration range of the cadmium (i.e. below 6 mg/l) was dictated by the linear range of the AAS.

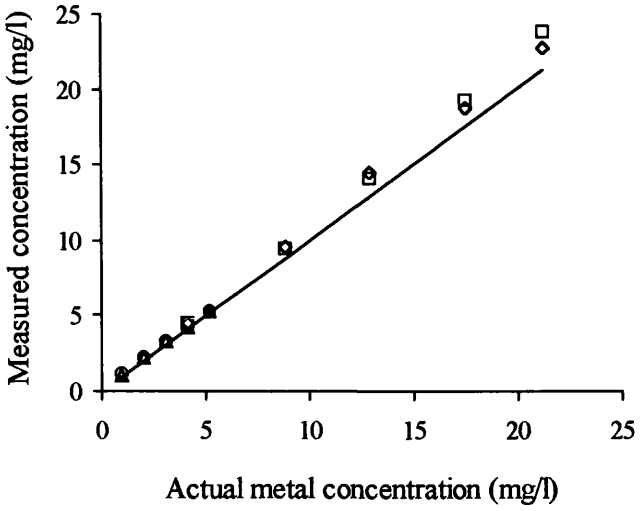

Figure 4.1 Assessment of the interference by bacterial culture media upon cadmium and nickel determinations by atomic absorption spectrometry. The line (—) represents metal solutions made up in water. Symbols show cadmium in (○) 13 g/l NB and (△) 5g/l tryptone; nickel in (□) 13 g/l NB and (◇) 5 g/l tryptone. [Error bars of standard deviations, which were better than SD ±0.1 for all data, are omitted for clarity.]

The graph shows that cadmium analysis is not affected by the presence of growth broth. The estimate of nickel however is affected by both NB and tryptone in the metal solution. At a true metal concentration of 20.0 mg/l the AAS analysis detected 22.2 mg/l in the presence of nutrient broth, and at 10.0 mg/l detected 10.9 mg/l. The difference was not so marked at the higher concentrations in the presence of tryptone, for at 10.0 mg/l, 10.9 mg/l were detected, and at 20.0 mg/l, 21.5 mg/l were detected. The amount of metal detected is falsely enhanced since no extra nickel has been introduced into the system. Analysis of the growth media by inductively coupled plasma (ICP) detected no nickel in the growth media or water. It is clear that the enhanced nickel concentration is an aberration due to the interference with analysis.

The divergence from the true metal concentration, although significant for determinations made in NB (using the *t*-test at a probability level of 5%), was not very large when considering that the effect is due to a relatively concentrated broth solution. The experimental protocol (Section 3.2.8) describes how the biofilm was developed and the isotherm data collected. It is apparent that under such a regime the metal solutions could only be contaminated either by a small volume of broth

remaining after rinsing or small quantity of media components which may have adsorbed to the carbon, only to desorb into the metal solution. Consequently the effect of a dilute solution of broth, such as that which may be carried over, was quantified.

4.1.1.2 By dilute growth broths and carbon leachates

It is known that desorption of surface attached groups can occur upon wetting. The pH of the wetting solution undergoes an alkaline pH shift which is due to a hydrolytic reaction, with the evolution of hydroxyl groups. The two carbons used in the study (F400 and Picabiol) originate from different raw materials (the former coal, the latter, wood based). The carbon source, and the activation process employed by the different manufacturers, confers different adsorptive qualities on the carbon by producing diverse functional groups and an altered structure. So it is reasonable to assume that the groups leaching from the carbon are manifold, and with varying influence upon metal measurement. Thus the leachate from the carbon surface found in a distilled water test after an initial wetting with water was tested for its effect upon metal measurement by AAS.

Figures 4.2 a and b demonstrate the impact of the groups associated with the carbon surface which have leached into sterile distilled water, upon AAS analysis of known concentrations of the metals Ni(II), Cd(II) and Cr(VI).

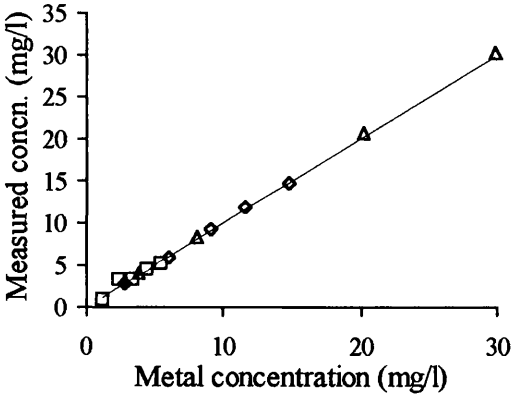

Figure 4.2a Variation between the true and measured concentration of metals (Ni (◇), Cd (□), and Cr (△)) caused by F400 leachate.

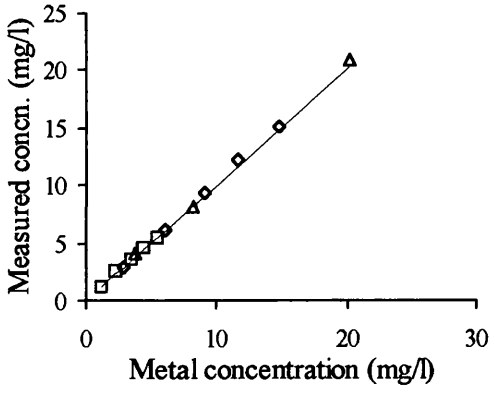

Figure 4.2b Variation between the true and measured concentration of metals (Ni (◇), Cd (□), and Cr (△)) caused by Picabiol leachate.

The measured points superimpose almost exactly on the line depicting the true concentration and the variation of all data points was better than SD ±0.1. The effect of the leached groups upon metal test solutions is negligible. The initial wetting water was analysed by ICP and, in support of these results, was found to contain only trace metals and specifically none of the test metals used in these experiments.

Further to the investigation of undiluted growth media was the quantification of the effect of dilute media components in conjunction with carbon leachates which result when carbon is incubated with a growth medium.

Figures 4.2 c and d demonstrate that atomic absorption spectrometric measurement of metal solutions containing carbon leachate and a small amount of broth components (comparable with that which may be carried over following the culture cycle) remained unaffected by these constituents. The accuracy of nickel, cadmium and chromium determinations in the range 5-30 mg/l was not compromised by components introduced into the liquid phase through the experimental practice.

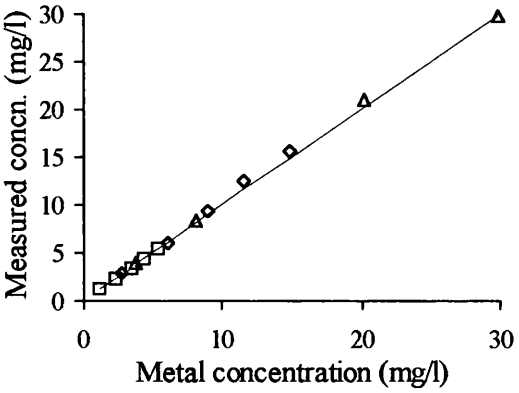

Fig. 4.2c The effect of leachate, from F400 incubated with tryptone, upon AAS quantification of predetermined metal solutions, Ni (◇), Cd (□), and Cr (△). [SD ±0.1].

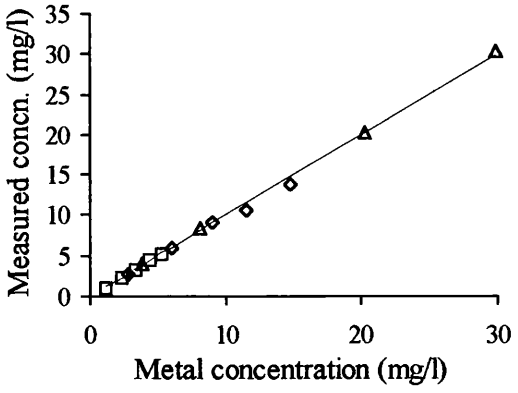

Fig. 4.2d The effect of leachate, from Picabiol incubated with tryptone, upon AAS quantification of predetermined metal solutions, Ni (◇), Cd (□), and Cr (△). [SD ±0.1].

4.1.1.3 By buffer

As mentioned in Section 3.1.3, Ringer's solution (¼ strength) would be a useful adjunct to the test metal solution. The buffer would aid cell homeostasis when the conditions surrounding the cells were altered by the removal of the culture solution and addition of the metal solution. The effect was quantified at the standard concentration of ¼ strength, since if used the metal would be made up in a solution of the buffer.

The digression, from the true metal concentration (shown by the solid line), of the metal solutions measured by AAS (Cd(II), 1-5 mg/l; Ni(II), 5-25 mg/l and; Cr(VI), 5-25 mg/l) in the presence of Ringer's solution is shown in Figure 4.3.

The graph suggests that the measurement of nickel and cadmium is affected little by the presence of ¼ strength Ringer's solution in the test medium, although some augmentation of nickel measurement is evident. At a nickel concentration of 20.0 mg/l the analysis detected 21.3 mg/l (a 6.7% enhancement), a 5.0 mg/l solution produced a 7.2% enhancement; this is a small but significant deviation (using a *t*-test at 5% probability) from the true metal concentration.

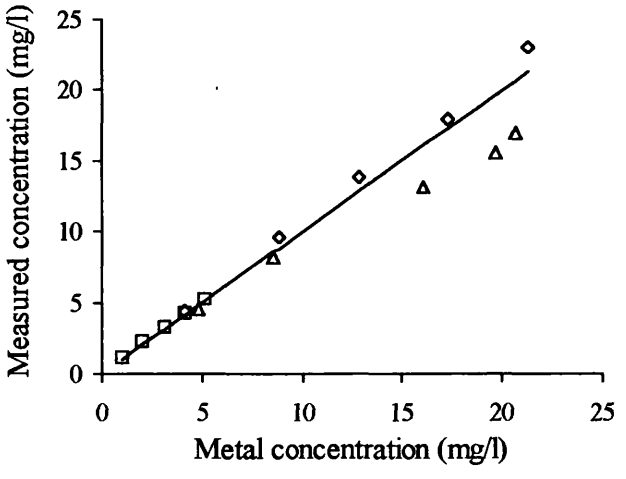

Figure 4.3 The effect of ¼ strength Ringer's solution upon AAS analysis of known concentrations of Ni (◇), Cd (□), and Cr (△). [SD ±0.3].

Sensitivity to chromium was significantly depressed by the presence of Ringer's solution, obvious particularly at the higher metal concentrations. At 20.0 mg/l there was 20% signal suppression, whereas at 5.0 mg/l Cr (VI) there was no suppression.

Ringer's solution (¼ strength) had a different effect upon the detection of each of the three metals. Chromium was the most affected, notably at the higher concentrations. Although the effect of Ringer's solution is quantifiable, the magnitude of the variation, and the differential between the three metals would cast doubt upon the validity of results should the buffer be included in the metal medium. Ringer's solution is also likely to have some effect upon the adsorption capacity of the carbon.

Similarly phosphate and tris/HCl buffers were discarded since their effect upon adsorption capacity and interference with AAS analysis was found to out-weigh the benefits of their use within the system.

4.1.2 The effect of growth media on carbon adsorption capacity

Growth media had a positive effect upon the removal of cadmium and nickel from solution (see Section 4.1.1.1), however the addition of growth medium to a system testing for chromium uptake by carbon, suppressed accumulation of the metal. The carbon's capacity was further reduced in the presence of biofilm. The potential growth media were tested in order to find a nutrient source suitable for culture of the biofilm and with little or no negative effect upon carbon adsorption capacity.

Nutrient broth, tryptone, yeast extract and di-ammonium hydrogen citrate ($(NH_4)_2HC_6H_5O_7$), at two concentrations (5 g/l and 10 g/l), were assessed for their effect upon the metal capacity of F400. The results are summarized in Table 4.1. The capacity of the carbon for Cr (VI) (at a concentration of approximately 20 mg/l), following a 7 day incubation with the medium, was expressed as a percentage of the maximum uptake achieved when control samples of carbon were incubated in water alone.

Table 4.1 Media effects upon the chromium (VI) capacity of carbon F400

Culture medium	Concentration (mg/l)	Average Cr(VI) uptake (% of maximum)
Nutrient broth	10	35
Tryptone	10	52
	5	61
Yeast extract	10	63
	5	65
$(NH_4)_2HC_6H_5O_7$	10	63
	5	63

A nutrient broth bathed carbon system provided poor conditions for chromium adsorption; the uptake established was only 35% of that achieved when the carbon was contacted by water alone. Generally tryptone (at 5 g/l), yeast extract and di-ammonium hydrogen citrate affected the Cr(VI) adsorption by carbon to the same extent: approximately 65% of the possible uptake was achieved in the presence of these media.

Di-ammonium hydrogen citrate although affecting the Cr(VI) uptake little, was not an effective growth medium for cells; requiring growth supplements, amino acids and vitamins in order to produce a measurable biofilm. Yeast extract, although facilitating marginally greater Cr(VI) uptake than tryptone, suffered from poorer uniformity of composition (Anon., 1990), hence coupled with the evidence from Section 4.1.1.1, tryptone, at a concentration of 5 g/l, was used for development of biofilm in the subsequent isotherm studies. Other components of nutrient broth such as peptone were not chosen due to their high salt concentration and their effect on AA spectrometry (Section 4.1.1.1).

4.1.3 Rinsing regimes

Rinsing cycles of length between 1 and 24 hours, with up to 3 repeats were tested in an effort to reduce the adverse effect of growth media on chromium uptake. Rinsing

always improved metal uptake. Rinses of duration 1 hour were the least effective. Recovery in the adsorption potential of carbon improved with increasing incubation time in water, and repeated rinses. In excess of 95% of chromium was removed from a 20 mg/l solution by carbon not exposed to growth media.

Carbon exposed to growth media and unrinsed before the metal solution was added, removed only 60% of the Cr(VI) from solution. One hour rinses initially regained a further 5% capacity, with increments of only 1% with successive rinses. Rinses of 24 h each were most effective; 71% uptake was achieved with one rinse; each successive rinse recovered approximately 3% uptake, rising to a maximum of 76% after the third rinse with sterile distilled water.

Repeated rinse cycles were not employed because of the added risk of contamination upon opening reaction vessels which may have contained sufficient nutrients to support growth. An assay of biofilm protein following repeated rinse cycles indicated less protein and hence less cells on the carbon, suggesting that the rinsing regimes were deleterious to maintenance of the biofilm.

A compromise of three rinses with 5 ml of sterile distilled water immediately before metal addition was employed to remove gross carry-over of tryptone into the metal test solution without subjecting the biofilm to conditions which may cause its breakdown.

4.2 Bacterial resilience to metals

4.2.1 Metal resistance of planktonic cells

An initial screening of the suitability of the short-listed bacteria for metal studies was made by means of planktonic cultures. The resistance of three of the chosen bacteria to the metal solutions containing Cr(VI), Ni(II) and Cd(II), was assessed by means of growth tests. Triplicate culture flasks were inoculated with an overnight culture of the test organism and the proliferation was monitored for 8 days. Growth was assessed visually by the turbidity of the broth. The results are summarized in Table 4.2.

Table 4.2 Growth of planktonic cells in single component metal solutions.

Micro-organism	Metal concentration (mg/l) at which growth occurred within 8 days		
	Cr(VI)	Ni(II)	Cd(II)
K. pneumoniae	<30	<50	<30
E. aerogenes	<30	<100	<50
Gram-negative isolate	<50	<50	-

In all but the very lowest concentrations (i.e. 5 mg/l Cr(VI) and Cd(II), and 10 mg/l Ni(II)) the lag phase increased over that of the control cultures grown without metal, which generally became turbid to the naked eye (i.e. containing around 10^7 cells/ml) within 8-24 hours. Growth was only achieved at concentrations up to those noted in the table, and proliferation was suppressed by metal concentrations at and in excess of those shown. *Enterobacter aerogenes* grew in concentrations of Ni(II) below 100 mg/l and in Cd(II) at up to 50 mg/l within the 8 day incubation period and was generally the organism most tolerant to the three metals, although the Gram-negative rod isolated from a spent GAC column was more tolerant of Cr(VI). The unclassified isolate from a water treatment column proliferated at Cr(VI) concentrations of up to 50 mg/l but was less tolerant of Ni(II) than the leading organism. *Klebsiella pneumoniae* was generally less tolerant than the other organisms, and grew in Cr(VI) and Cd(II) at concentrations only up to 30 mg/l, and Ni(II) at up to 50 mg/l.

After eight days the flasks remaining non-turbid were re-incubated. When a total of 28 days had elapsed all of these had failed to become turbid and so it was assumed that metals at and above the concentrations noted in Table 4.2 were lethal to the cells.

4.2.2 Optimization of culture age for metal accumulation

That *K. pneumoniae* cultures of 7-9 days were the most effective at accumulating Cr(VI) can be seen from Figure 4.4. Additional incubation after a culturing period of

9 days neither increased cell numbers nor improved uptake capacity. The equilibrium cell population reached a maximum after 1-2 days of culture, their better survival demonstrating that the young cells are more resilient to Cr(VI), contrastingly metal uptake is at a minimum of between 23 and 28%.

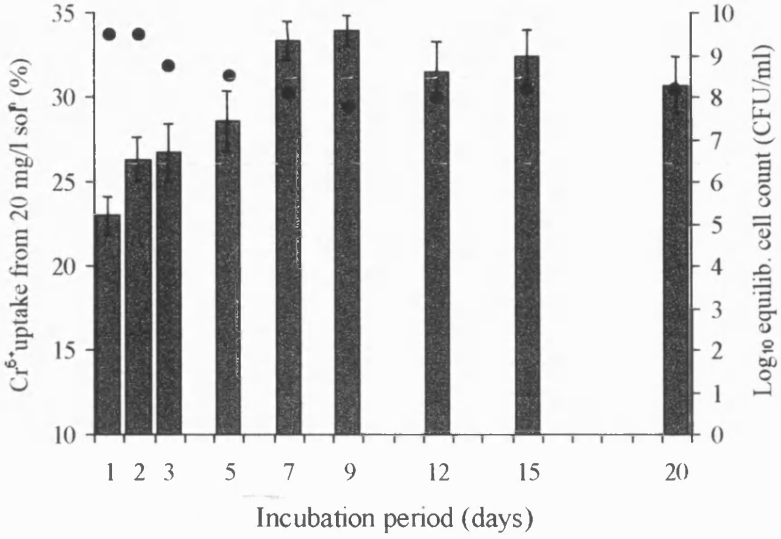

Figure 4.4 Optimum culture age for Cr(VI) accumulation, bars (▬) depict the percentage uptake by *K. pneumoniae* from a 20 mg/l solution of chromium, points (●) show the cell count (in CFU/ml) of viable bacteria after 3 days contact time, when an equilibrium of metal uptake had been reached.

These observations may reflect the metal resistance mechanisms of bacterial cells. Mechanisms employed by young cells may rely upon metabolic activity and active transport to exclude metal from the cells and maintain homeostasis. Older static cells do not sustain this active exclusion of metal, and so the cell population declined after 7 days to ~ 10^8 CFU/ml. An alternative mechanism of uptake may be by passive accumulation at the cell periphery (envelope). Older, starved cultures may produce more of the exopolysaccharide matrix (Sutherland, 1982) highlighted in previous studies as a good accumulator of metals. This too is a defensive mechanism; the metal is immobilized outside the cell where its effects upon the cell are less marked. This mechanism does not rely upon metabolic activity and so is not expensive to maintain by cells in stationary phase. Young cells, having little EPS, would thus be less efficient at accumulating metal.

4.2.3 Metal accumulation by planktonic cells

Metal accumulation tests were run in conjunction with the growth experiments. The organisms sought were required not only to be tolerant of metals, ultimately surviving in a waste-water GAC treatment column, but also to augment the removal of potentially toxic metals from the waste stream. Thus the metal sequestering capacity of each of the test organisms is presented in Table 4.3. A 7 day culture was challenged with a 20 mg/l metal solution and at equilibrium the metal remaining in a centrifuged and filtered sample was compared with similarly treated control samples without bacteria.

Table 4.3 Comparative accumulation of metal by 7 day axenic cultures.

Micro-organism	Percentage removal of metal from 20 mg/l solution		
	Cr(VI)	Ni(II)	Cd(II)
K. pneumoniae	20-30	30-35	40-50
Z. ramigera	6	0	-
E. aerogenes	0	0	25
Gram-negative isolate	0	*4	-

* Uptake statistically insignificant when compared with control flasks without organisms.

Only one of the bacterial strains, *Klebsiella pneumoniae* subsp. *pneumoniae* was able to accumulate chromium, nickel and cadmium. Of these metals Cr(VI) was the least successfully accumulated, with only 20-30% of the metal having been removed from solution by the cells. *Klebsiella pneumoniae* demonstrated a particularly high capacity for Cd(II) and between 40 and 50% of the 2 mg cadmium available in solution was removed by the cells. Approximately 30% of Ni(II) was removed from solution. The remaining organisms each only accumulated one of the metals; *Zoogloea ramigera* and the Gram-negative isolate removed Cr(VI) (6%) and Ni(II) (4%) respectively.

Enterobacter aerogenes removed a considerable mass of Cd(II) from solution (i.e. 25%) but accumulated neither Cr(VI) nor Ni(II).

The 4% uptake mediated by the Gram-negative isolate was so small as to be statistically insignificant when a comparison was made between the variance about the mean of the test and control samples.

Sonication of cell samples which had accumulated metal, followed by centrifugation and filtration to remove cell debris before analysis of the supernatant demonstrated that much of the accumulated metal could be recovered through sonication. All of the 22% chromium removed by a culture of *K. pneumoniae* from a 20 mg/l solution of the metal was recovered from the cells by sonication.

4.2.4 Rate of attainment of equilibrium

The rate of attainment of equilibrium between adsorbed chromium and that remaining in solution is depicted in Figure 4.5.

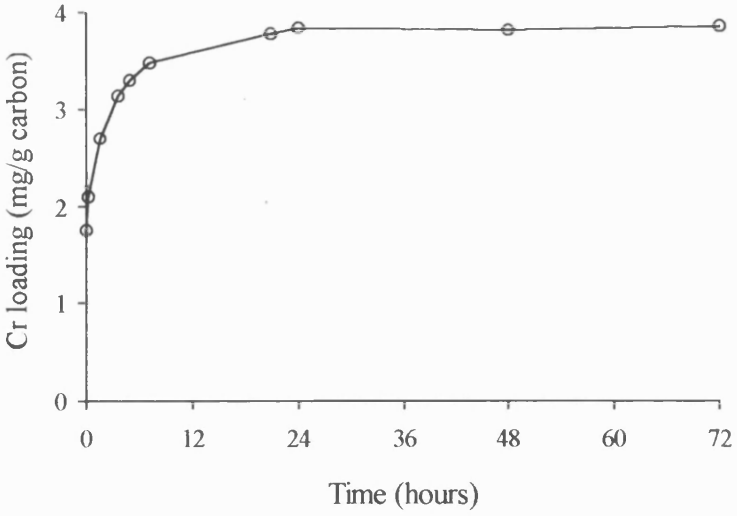

Figure 4.5 The rate of adsorption of Cr(II) by 0.5 g F400 from a 20 mg/l solution.

An equilibrium was reached within 24 hours, and the rate of uptake was at its greatest within the first 60 minutes. Fifty percent of the mass of Cr available in solution was removed by the carbon at equilibrium. This amounted to a loading of 3.9 mg Cr per

gram of activated carbon, from the 200ml of solution of concentration 19.64 mg/l measured in the parallel flask at time = 0.

Results

5.1 Carbon porosity and surface area studies

Information upon the porosity and surface area of the carbons was gained using the technique of mercury porosimetry. The volume of mercury entering the sample with each increase in pressure was accurately measured by a penetrometer within the porosimeter. The volume of mercury within the sample at any value of applied pressure gave the volume of all pores having a diameter equal to or greater than the calculated pore radius for that pressure (using the standard pressure table 10). The data are presented in Section 5.1.1 as intrusion/extrusion curves, Section 5.1.2 shows pore size distribution, and 5.1.3, a comparison of carbon surface areas. A summary of the porosity statistics is given in Section 5.1.4.

5.1.1 Mercury intrusion/extrusion curves

The contrasting intrusion/extrusion curves of F400 and Picabiol and anthracite are presented. All intrusion volumes presented on the graphs are in millilitres of mercury per gram of carbon.

5.1.1.1 Filtrasorb F400

The intrusion/extrusion curve (Figure 5.1) for Filtrasorb 400 (F400) is typical of a porous solid and is a characteristic Type IV isotherm. Such an isotherm is consistent with a porous adsorbent possessing pores in the radius range of approximately 15-1000 Å (0.0015 - 0.10 μm). The initial pressure of 0.47 psi produced no intrusion, but as the pressure was increased by only 1 psia, the volume of mercury taken up by the sample increased rapidly (to 0.2 ml/g carbon) this was due to the filling of the large

interparticle voids. As pressure was increased from 3 psia to 65 psia (pore diameters 60-3 μm) the gradient of the intrusion graph became flatter, denoting only a small amount of porosity in this range.

A steady increase in the volume of mercury entering the sample was noted as the maximum pressure of 60,000 psia was approached. This corresponded to the small pores (in the range 3 μm-0.003 μm (3×10^4 - 30 Å)) within the carbon particles filling with mercury.

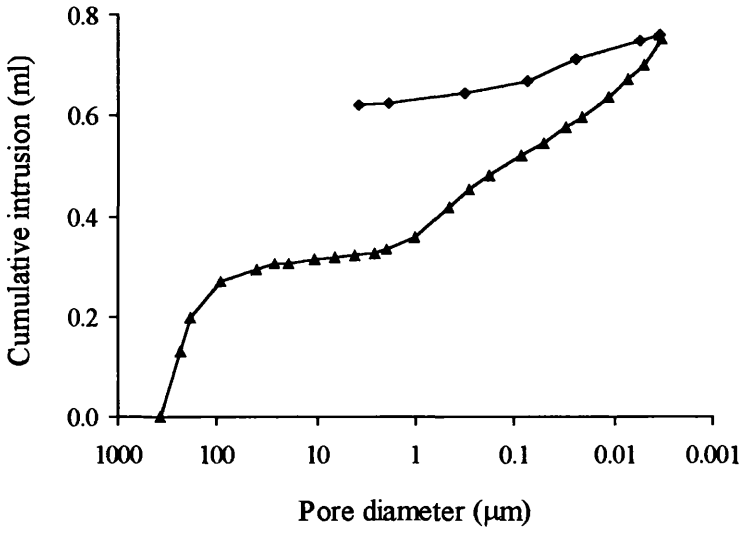

Figure 5.1 The mercury intrusion (▲)/extrusion (◆) curve for F400 porous activated carbon.

Typically the intrusion/extrusion curve exhibits hysteresis; the path followed by the extrusion curve is not the same as the intrusion path. The volume indicated on the extrusion curve, at a pressure of 3000 psia for example, was always higher (i.e. 0.68 ml/g carbon) than the intrusion volume (0.54 ml/g) at the same pressure. Mere relaxation of the pressure conditions did not supply a driving force to overcome the interactive forces formed between the carbon surface and the mercury molecules following intrusion. Work must be expended to overcome this pore potential, hence after the first intrusion/extrusion cycle some of the mercury was retained by the sample preventing the loop from closing. Subsequent intrusion cycles would continue

to show hysteresis until reaching a point where no further mercury entrapment occurred and the loop would finally close.

5.1.1.2 Picabiol

The Picabiol mercury intrusion/extrusion curve (Figure 5.2) was atypical of a porous substance; the extrusion curve apparently lying below the intrusion curve and demonstrating an 'inverted hysteresis'. Since this was likely to be an artefact, reasons were sought for the occurrence, and the porosity data interpreted accordingly.

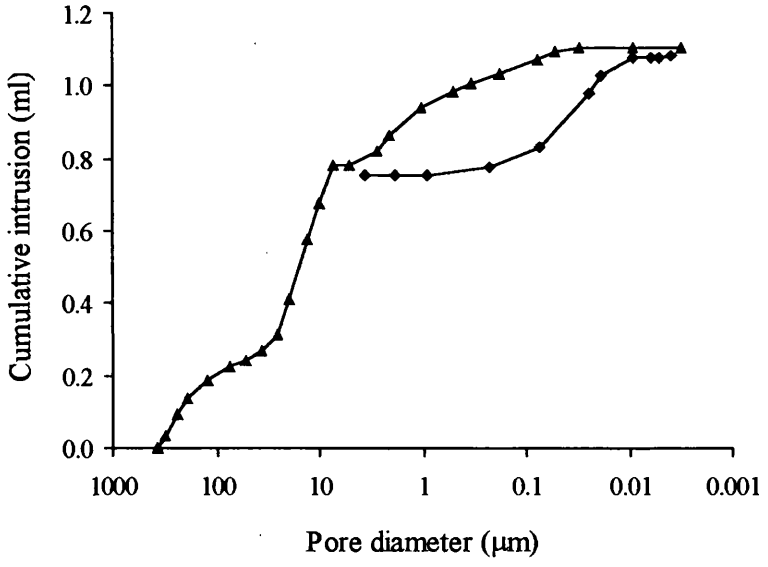

Figure 5.2 The mercury intrusion (▲)/extrusion (◆) curve for the porous activated carbon Picabiol.

At an intrusion pressure of 25 psia (corresponding to a pore diameter of 7 μm) the gradient of the curve tended to zero and no intrusion took place until the pressure had increased by a further 10 psi. This may be indicative of a lack of pores at this diameter (which may be seen more clearly in Figure 5.6) or could be a result of some change in the carbon structure as the pressure of intrusion was increased. This is also borne out by the unusual position of the extrusion curve. In a carbon sample unaffected by high pressure, the extrusion curve would always be above the intrusion curve (as Figure 5.1) indicative of the larger volume of mercury remaining in the carbon even at the same pressure as the intrusion. The lower extrusion curve of Figure 5.2 indicates that extrusion of mercury was easier than its intrusion at the same pressure and less work

was required to remove it than was normal. This suggested that some crushing or compaction of small pores had occurred, forcing mercury back into the larger pores by displacement, then to be released more easily as the pressure was relaxed. Alternatively the wall structure of the pores may have broken down, forming larger pores from which the mercury could escape more freely.

5.1.1.3 Anthracite

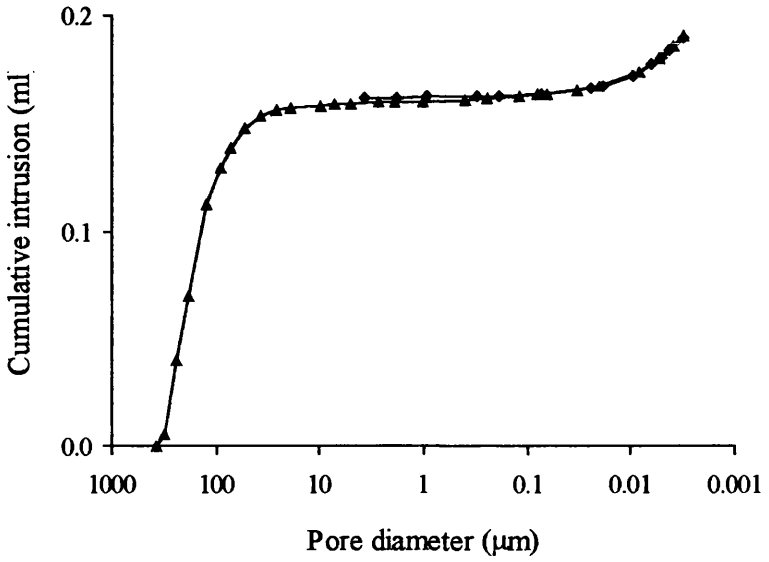

Figure 5.3 The mercury intrusion (▲)/extrusion (◆) curve for non-activated anthracite.

The anthracite intrusion/extrusion curve is standard in that it exhibits some small degree of hysteresis, however from the scale of the y-axis it can be seen that only a small fraction of the volume of mercury forced into the activated carbons was taken up by anthracite.

The initial mercury intrusion into the void space between particles was of a similar magnitude to that of the activated carbons, although the volume was smaller since the anthracite particle size (< 2 mm) was generally smaller than those of the other carbons (2-4 mm), leading to more efficient packing of the particles in the sample vessel and so less void space.

The intrusion, particularly into pores of diameter between 10.0-0.1 μm was negligible, characteristic of a substance with low porosity. That the pore volume, and hence

surface area, of the anthracite was low is also amply demonstrated in Figure 5.7. The cumulative intrusion curve and electron micrographs prove that anthracite is indeed a non-activated carbon with little porosity, a low surface area and few surface features.

5.1.2 Pore volume distribution

Graphs of incremental mercury intrusion with increasing pressure are presented for the three test carbons; these show the volume of mercury held within pores of each discrete diameter. Graphs are presented with the same ordinate (y-axis) scale to aid comparison of the three carbons.

5.1.2.1 F400

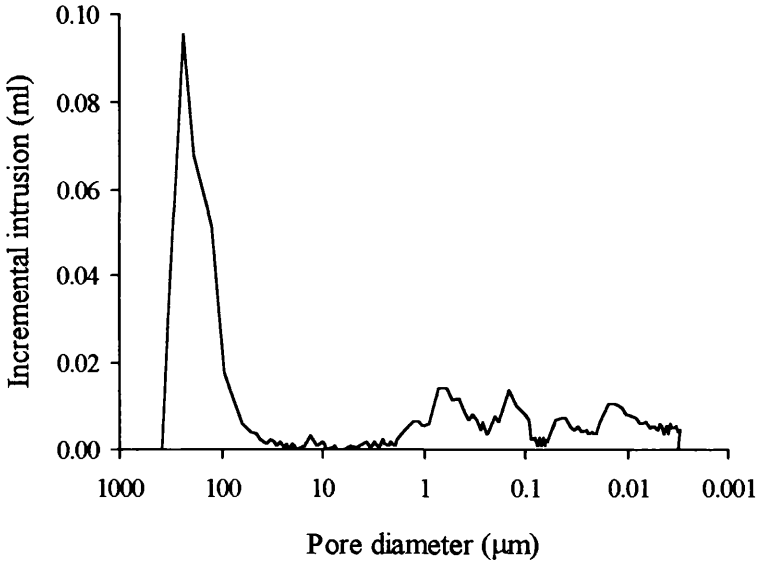

Figure 5.4 Volume of mercury held within pores of F400 activated carbon.

The initial pronounced peak, rising to an intrusion volume of almost 0.1 ml of mercury per gram of carbon was a result of the mercury, forced into the sample at low pressure, filling the inter-particle voids. This occurred between 0.5 psia and 7.0 psia, where the diameter of the voids/pores was from 380 μm to approximately 30 μm.

There were few pores of the size distribution 60-2 μm as can be seen from the small volume of mercury (the area below the graph) that was trapped within them. The volume held within pores of the next size range (2.0-0.003 μm) was greater than that held within the larger pores (60-2 μm), and is demonstrated by the much larger area

beneath the curve. The differential cumulative plot (Figure 5.8 a) also evinces the preponderance of small pores.

This carbon has not only a mesoporous structure but is also rich in micropores (i.e. 0.2-0.1 μm) although the technique of mercury porosimetry is not best suited for detection of these micropores, and so for a complete profile at this scale the technique of gas adsorption at lower pressure would be more usefully employed.

5.1.2.2 Picabiol

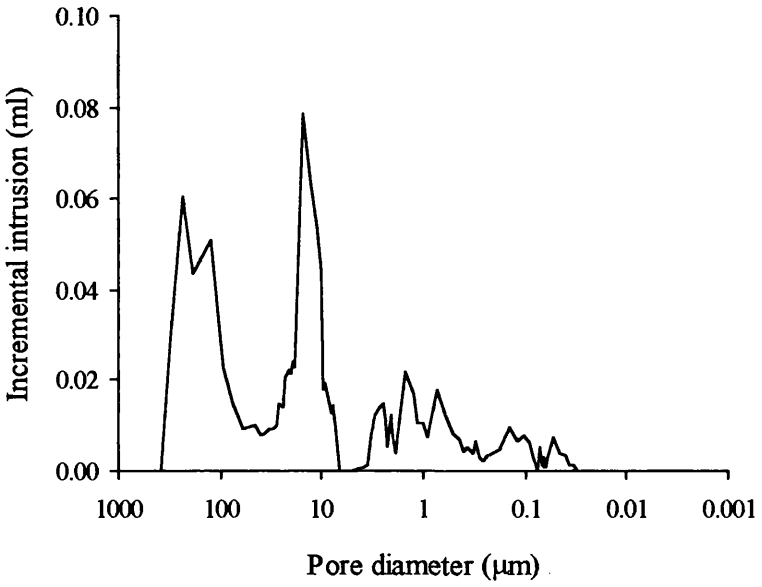

Figure 5.6 Volume of mercury held within the pores of the activated carbon Picabiol.

Once again the initial broad peak (between 380 and 40 μm) was produced as mercury filled the voids between carbon particles.

Pores common in Picabiol but lacking in carbon F400 were of the range of diameters 25-8 μm. Such pore sizes correspond closely with those visualized by scanning electron microscopy (Figure 5.10 b) and were produced from the cellular structure of the wood, still present even following activation. Surface features of this diameter were hypothesized to augment bacterial colonization (Section 6.3.2).

The zero gradient upon the cumulative intrusion plot (Figure 5.2) translates into a base line with no intrusion (at pore diameter 7-5 μm) possibly indicative of a total lack

of pores of this size, or perhaps an artefact caused by a change of the carbon ultrastructure as the pressure was applied. In the light of this minimum point of incremental intrusion, and the unusual extrusion curve, the high pressure data was interpreted rather loosely and not without confirmation by another source such as visualization by electron microscopy.

A much greater proportion of the total volume held within a particle was found in large pores of diameter between 40 μm and 7 μm (corresponding to an intrusion pressure of 4.5-25.0 psia) than was the case with carbon F400 where there were few pores of that size. Generally the pore size distribution of Picabiol was more limited than that of F400; there were none of the very small pores (i.e. ≤ 0.01 μm) characteristic of F400, and no pores were measured at less than 0.03 μm in diameter. A qualitative analysis of the surface topography of carbon by SEM confirmed that F400 has many microporous surface features, whereas at the same magnification Picabiol tends to smoothness (see Section 5.2.2 and Figures 5.10 a & b).

5.1.2.3 Anthracite

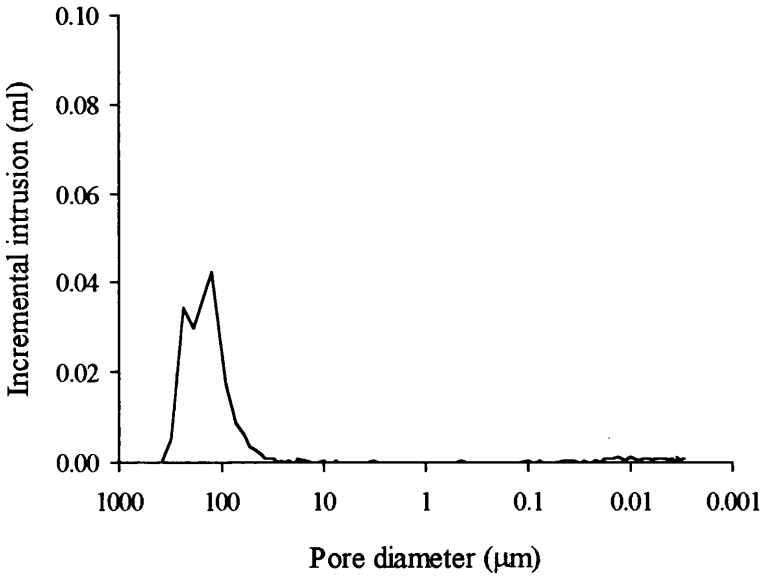

Figure 5.7 Pore volume distribution of the non-activated carbon anthracite.

The early anthracite peak around the 100 μm diameter was due to filling of the interparticle voids with mercury. Plotted with the same scale as the F400 and Picabiol

incremental intrusion curves it was clear that this non-activated carbon had only a fraction of the pore volume and surface area of its activated counterparts. Scanning electron micrographs (Figures 5.9 c and 5.10 c) confirm the smooth surface, characteristic of the anthracite suggested by this data.

A little enhanced porosity was found at the smallest pore sizes (< 0.02 μm), although these were too small to have made additional surface area available for bacterial attachment, and had an insignificant effect upon metal adsorption.

5.1.3 Pore surface area distribution

Figures 5.8 a and b depict the comparative surface areas of carbons F400 and Picabiol. The graphs show the same data but the second (Fig. 5.8 b), with an expanded y-axis, enables a detailed comparison of the surface areas within the pore size range 100.0-0.01 μm.

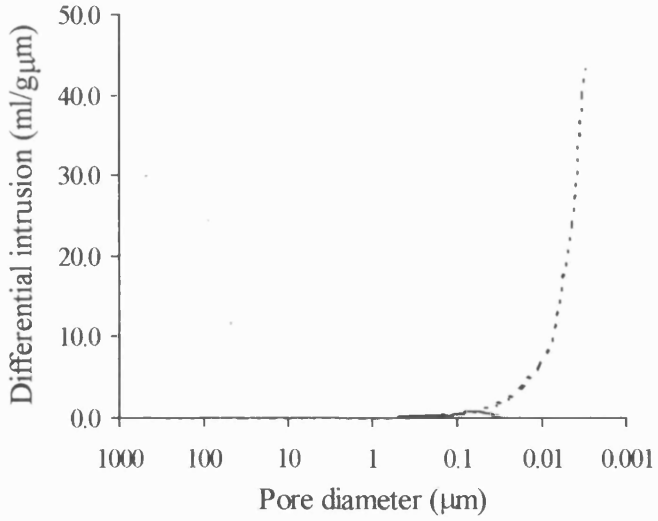

Figure 5.8 a The surface areas of F400 (- - - -) and Picabiol (—).

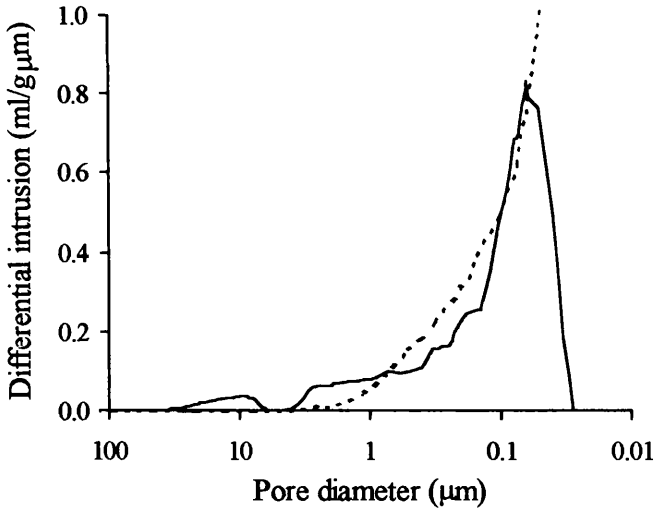

Figure 5.8 b The surface area of F400 (- - - -) and Picabiol (—).

Much of the surface area of activated carbon F400 is held within pores of size < 1 μm and indeed most of the extended surface area is from pores of < 0.01 μm. This is commensurate with manufacturers specification of F400 which is for a product of very great surface area, in the region of 1100 m^2/g, for efficient adsorption of organic compounds. In contrast the lignite based activated carbon, Picabiol, has a comparatively reduced surface area, much of which is solely held within pores larger than those of F400 i.e. greater than 0.25 μm. Although the majority of the surface area of Picabiol seems to exist within pores of between 4.5 and 0.03 μm, an isolated peak is noted at a pore size of approximately 10 μm (range 7-25 μm).

5.1.4 Comparative carbon porosity data

The porosity data for the activated carbons: F400 and Picabiol, and the non-activated precursor; anthracite, gained by the technique of mercury porosimetry are presented in Table 5.1.

The carbon with greatest pore area was F400, which is a reflection of the very large number of small pores, micropores of diameter < 2 nm, which it possesses. The high pore area and thus surface area confirms manufacturers claims for a carbon with characteristics of a high internal surface area and efficient removal of organics.

The larger mercury intrusion volume of Picabiol was not due to a greater porosity, for the total pore area can be seen to be only a small fraction of that of F400, but due to the larger particle size and lower density of Picabiol, causing it to take up more mercury in the void spaces and large pores, and the larger average pore diameter (some 34 times greater than the average pore diameter of F400).

The diameter of the 'pores' of anthracite appear to be comparatively small, but when taken in conjunction with the total intrusion volume, it can be assumed that these pores are simply tiny features on the particle surface rather than pores of an internal surface such as those found in an activated carbon.

Table 5.1 Summary of porosity statistics for the three test carbons.

Parameter	Carbon type		
	F400	Picabiol	Anthracite
Total intrusion volume (ml/g)	0.7593	1.1057	0.1903
Total pore area (m^2/g)	118.4	5.0	16.8
Average pore diameter (μm)	0.0257	0.8779	0.0452
Bulk density (g/ml)	0.620	0.381	1.140
Apparent (skeletal) density	1.170	0.658	1.455
Porosity %	47.0	42.1	21.7

5.2 Carbon surface characterization

Scanning electron microscopy (SEM) revealed the detailed surface characteristics of the three carbon, F400, Picabiol and anthracite. Two methods of visualization were used carbons were; a) gold sputter coated, viewed at ambient temperature, and b) viewed using cryogenic unit at -180°C and without a coating, in order to test that the results were comparable. The appearance of carbon was the same for each preparation method and so it can be assumed that the different techniques had no effect upon the carbon. Granules were randomly selected for microscopy, and even though only

relatively few could be examined by the technique, upon investigation the granules appeared to represent a typical sample of the carbon. Scanning electron micrographs of virgin carbons are presented in Figures 5.9 & 5.10 (a-c).

5.2.1 Granule characteristics

Low magnification (50-fold) cryo-scanning electron microscopy revealed the appearance of individual carbon granules and something of their structure (Figures 5.9 a-c).

5.2.1.1 F400

F400 granules (Figure 5.9 a) were large (between 2 and 3 mm at their largest dimension). The carbon possessed an uneven surface with a rough appearance but rounded edges; typical of bituminous coal based carbon.

5.2.1.2 Picabiol

Granules of Picabiol were generally the largest of the three carbons (range 2-4 mm in diameter), and were flatter and squarer than granules of F400. The structure of the carbon granule was already apparent at low magnification (Figure 5.9 b), and it is clear that the cellular structure of the raw material, wood, survives the carbonization and activation process and confers the parallel linear vein structure of the plant vascular bundles upon the carbon. Thus the carbon has a rough corrugated or ridged appearance.

5.2.1.3 Anthracite

Anthracite granules (Figure 5.9 c) were small (< 2 mm in diameter), angular and smooth surfaced with facets caused by the grinding process of granule formation. Anthracite is a coal based carbon which has not undergone the industrial activation process.

5.2.2 Carbon surface characteristics

The higher magnification (samples were magnified 500-fold, and the micrographs enlarged a further 3-fold) micrographs (Figures 5.10 a-c) aid in characterization of the carbons, enabling a comparison of the nature of the pores produced from different raw materials and the carbonization and activation processes. At higher magnification the ultrastructure of carbon became visible and the very different nature of each carbon was demonstrated as the detail was revealed.

5.2.2.1 F400

In Figure 5.10 a the F400 surface remains rough and irregular at high magnification. The pores are not the obvious cylindrical openings of text books, but rather crevices and interstices within the carbon plates. At still higher magnification (up to 15,000 times magnification) the surface continues to exhibit the same appearance, demonstrating its fractal nature.

Using the electron micrographs in conjunction with the porosity data (Figure 5.8 a) it can be concluded that much of the F400 porosity extends from the irregular carbon surface through the tiny surface features shown by electron microscopy and measured by porosimetry at the highest resolution (highest pressure/smallest pore size) of the equipment.

The majority of surface features of carbon F400 are therefore minute and held within pores or fissures much smaller than the size of a bacterial cell i.e. 1-2 μm in length.

5.2.2.2 Picabiol

At high magnification the ridge-like appearance of carbon Picabiol may be seen in more detail (Figure 5.10 b). The parallel sides of the 'troughs' are arranged at 30 μm intervals corresponding exactly to the diameter of xylem and phloem vessels within wood of living plants.

The carbon surface between these troughs is smooth when compared to the surface of F400 at the same magnification (as the walls of the cells would have been in life), and so provide little of the extended surface area of that carbon. Hence a greater

proportion of the surface area of these large flat granules are available for bacterial attachment (See Section 5.3). The pores near the centre of the micrograph are the communication 'pores' of the plant's secondary xylem vessels called sieve plates, here 10 μm x 4 μm.

Porosity data (Section 5.1) concurs with the qualitative SEM information. The Picabiol pore volume distribution (Figure 5.6) and surface area (Figure 5.8 b) demonstrate a peak in the population and confirm the pore sizes between 30 and 10 μm in diameter. At very high magnification resolution of the micrograph was lost easily due to the featurelessness of the carbon, a qualitative assessment resulted in the estimation of a smoother surface with less features than had the F400.

Viewed in cross section (Figure 5.16) carbon had a honeycomb appearance; the whole structure was composed of layers of these transport vessels. Entry to the interior of the granule could be gained via these long and wide parallel sided pores.

5.2.2.3 Anthracite

Under high magnification (Figure 5.10 c) the structure of anthracite remained consistent with its appearance at low magnification. The surface was smooth, with only faint undulations caused as the carbon was cleaved into granules. The edges of the faces thus formed are well defined and sharp. Once again the pictorial information corresponds with porosity data; mercury porosimetry found few pores and calculated surface area was very low; the total pore area was 16.8 m^2/g.

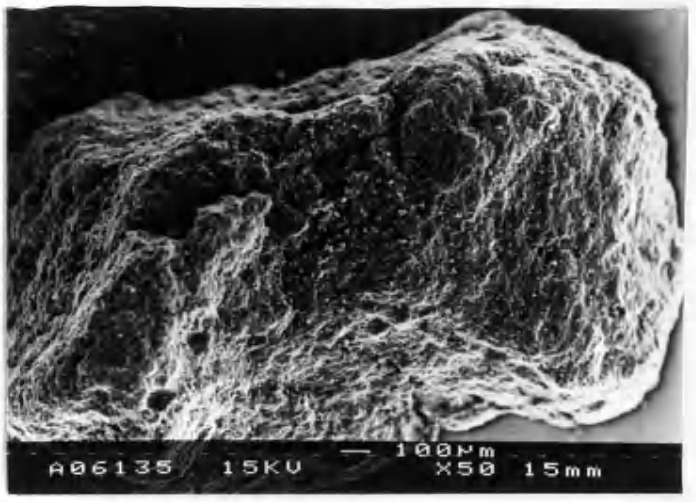

Figure 5.9 a *F400*, a bituminous coal based activated carbon, shown by cryo-scanning EM at low magnification. This typical granule, was of length 2.8 mm and possessed a characteristic roughened surface.

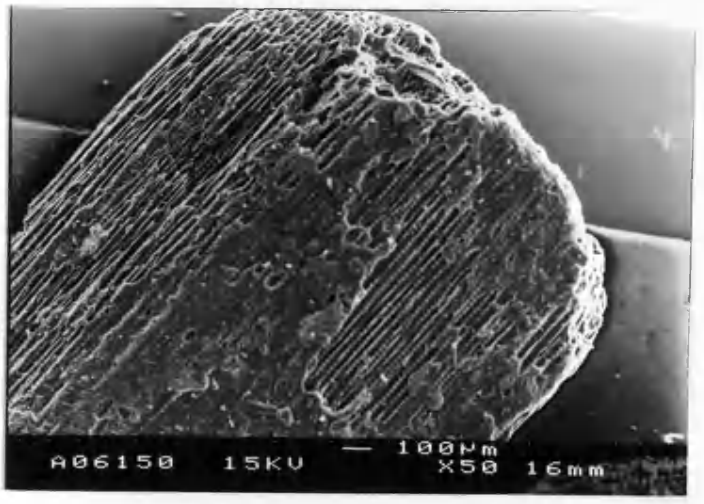

Figure 5.9 b *Picabiol*, under a low power magnification, had a distinctive cell structure originating from the wood based raw material. The granules were large, that depicted being 3.15 mm in length.

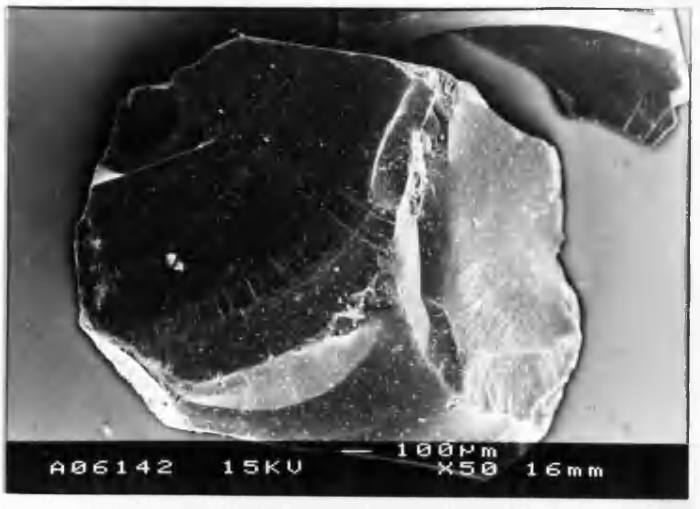

Figure 5.9 c The *anthracite* granule shown (length 1.92 mm) was viewed by scanning electron microscopy with cold stage. At low power the non-activated carbon appeared smooth surfaced and angular.

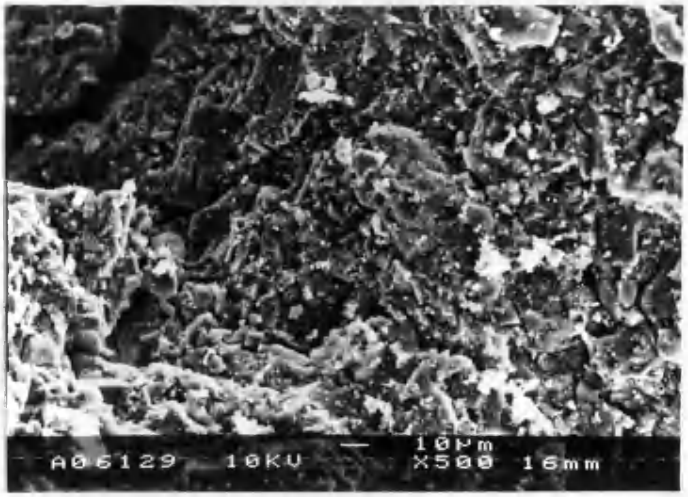

Figure 5.10 a Micrograph of *F400* activated carbon, unstained and viewed using a cryo-SEM. High magnification shows interstices between the carbon plates and demonstrates the fractal quality of carbon structure.

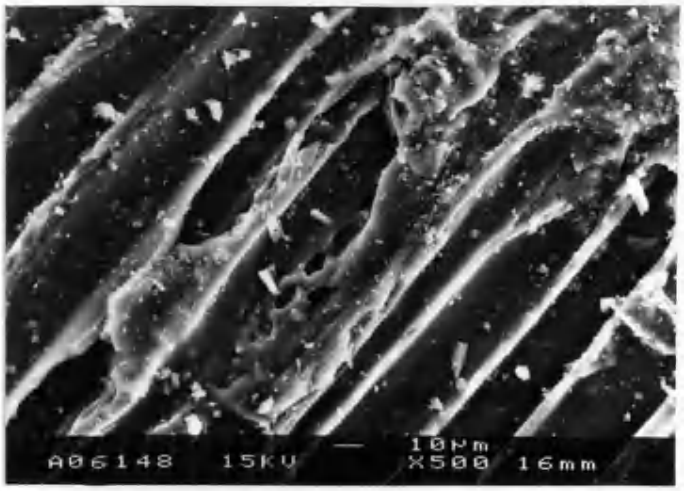

Figure 5.10 b *Picabiol* carbon viewed by cryo-scanning electron microscopy. The cellular structure of this wood based carbon became apparent particularly at high magnification.

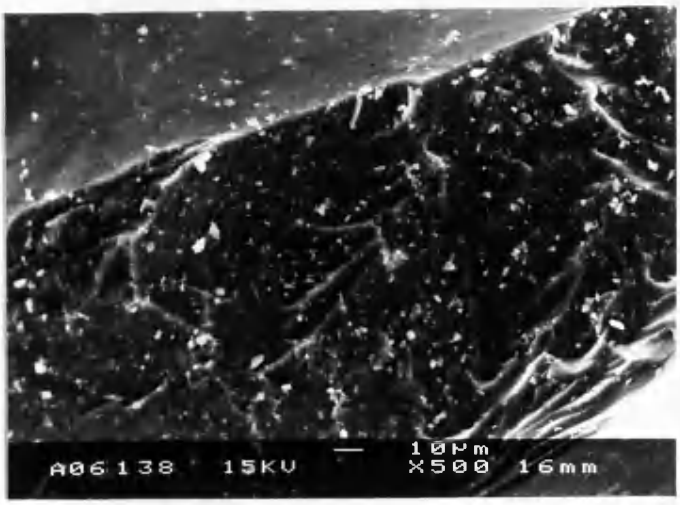

Figure 5.10 c The electron micrograph of *anthracite* shows that even under high power magnification the surface of uncarbonized and non-activated carbon was relatively smooth and featureless.

5.3 Biofilm studies

The quality, coverage, and mode of bacterial colonization of carbon was assessed after visualization by scanning electron microscopy. Biofilms were developed over GACs in shake flasks. Cells of *Agrobacterium radiobacter*, *Pseudomonas elodea*, and *Klebsiella pneumoniae* subsp. *pneumoniae* were compared. The organisms themselves had different morphologies. *Agrobacterium radiobacter* cells (Figures 5.11 & 5.12) were long narrow bacilli of size 2 x 0.5 μm. *Pseudomonas elodea* cells appeared to be shorter and broader, approximately 1.75 x 1 μm, although this may be due in part to the blanket of exopolysaccharide causing the cell boundary to be indistinct (Figure 5.13). Cells of *K. pneumoniae* (Figures 5.14-5.19) appeared by SEM to be the smallest of the three organisms examined, they were short, roundish rods with dimensions of 1.5 x 1 μm, and produced a rather thinner covering of exopolysaccharide than did *P. elodea*.

5.3.1 Preparation techniques

Different preparation techniques were employed to find which was the most suitable for qualitative analysis of biofilm using scanning electron microscopy (SEM).

5.3.1.1 Chemically fixed

Figure 5.11 shows a colony of *A. radiobacter* on the carbon surface. The cells, although exopolysaccharide producers are not swathed in a glycocalyx, and may be seen clearly as individual cells arranged in a disorderly fashion. Exopolysaccharide does not appear because the fixing and staining process, which utilizes glutaraldehyde and osmium tetroxide, fixed cells but stripped away exopolysaccharide. The carbon surface can still be seen at the periphery of the colony.

5.3.1.2 Freeze dried

Agrobacterium radiobacter is shown again in Figure 5.12 but the cells have a different appearance owing to the freeze drying process used in its preservation. The cells have a lattice or net-like three dimensional appearance, partially clothed with exopolysaccharide. This may not be indicative of an expanded network through which

the solution can circulate, as suggested by some authors, but rather is an artefact of the freeze drying process, which can cause shrinkage of the hydrated components.

5.3.1.3 Cryo-preserved

Cryo-preservation and use of a cold sample stage at -180°C has enabled a more life-like representation of *Pseudomonas elodea* upon the surface of F400 (Figure 5.13). The cells appear as indistinct bumps in a semi-random arrangement (probably following the contours of the carbon) beneath a blanket of exopolysaccharide. Care was taken during preparation, for if sublimation of ice crystals is continued for too long the sample can dry, and take on an appearance similar to freeze dried samples from which much of the moisture has been removed.

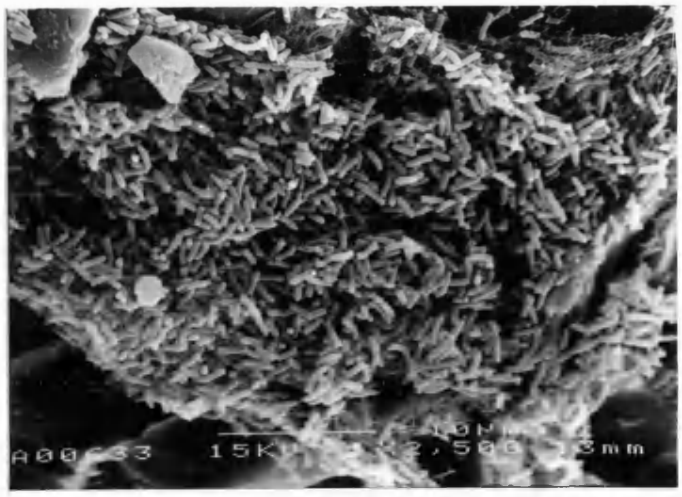

Figure 5.11 A micro-colony of *Agrobacterium radiobacter* upon carbon, prepared by chemical fixation and critical point drying.

Figure 5.12 Freeze dried sample of *Agrobacterium radiobacter* cells in a biofilm on carbon.

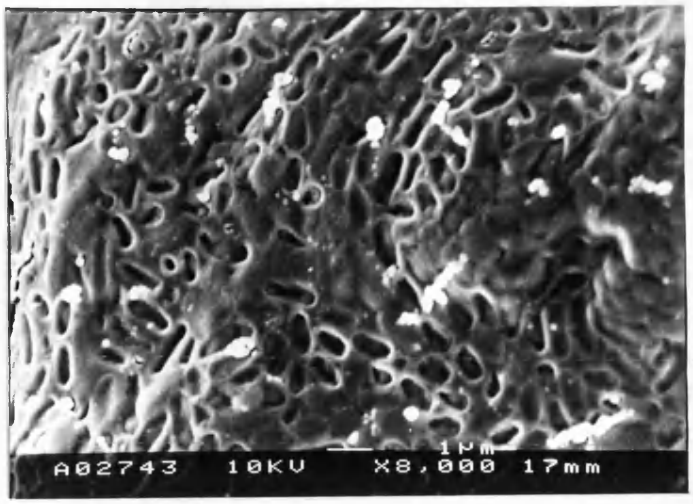

Figure 5.13 A biofilm of *Pseudomonas elodea* over F400, cryo-fixed and viewed on a cold stage at -180°C.

5.3.2 Carbon colonization

Klebsiella pneumoniae colonization of Picabiol and F400 is shown in Figures 5.14-5.17, and Figures 5.18-5.19 respectively.

5.3.2.1 Picabiol

Figure 5.14 depicts a crevice in the surface of Picabiol. Cells from a 3 day culture of *K. pneumoniae* became lodged in the crevice, and attached themselves away from the movement of the bulk solution. Small colonies are seen on the flat plain of the carbon (top left), but those cells within the fissure had insufficient space for proliferation and so are found only singly.

Figure 5.15 shows colonization of a longitudinal section of the carbonized secondary xylem vessels of the wood based carbon by a 3 day *K. pneumoniae* culture. The vessel runs left to right with a side wall shown bordering the top of the micrograph. From the nature of the colony formation it appears that cells attach in the shelter of the wall (shown in Figure 4.13 b), spreading first along the length of the wall, and then branching perpendicularly, across the smooth region between the walls. In this way the biofilm formed a patchy mosaic across the flatter regions of the carbon, leaving 'plaques' where there was no growth. The bacteria formed a monolayer of cells, covered with only a thin exopolysaccharide film such that individual cells are still

distinguishable. The plaques may fill with time but could remain if the surface is too smooth or exposed.

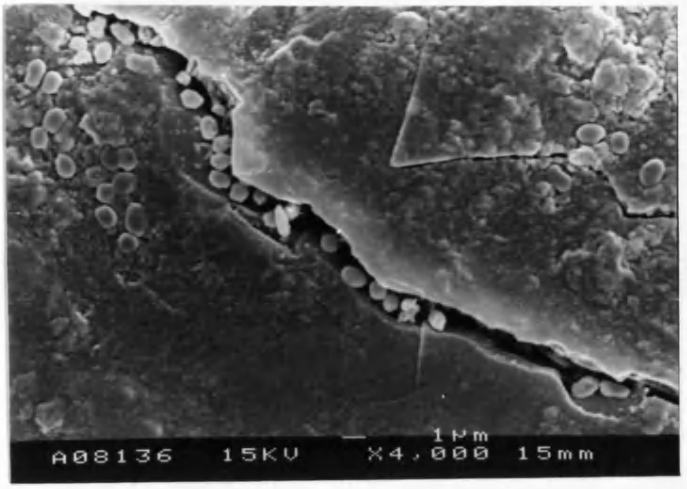

Figure 5.14 A cryo-fixed sample of Picabiol activated carbon with attached cells of *Klebsiella pneumoniae* from a 3 day culture upon the surface and within the crevice.

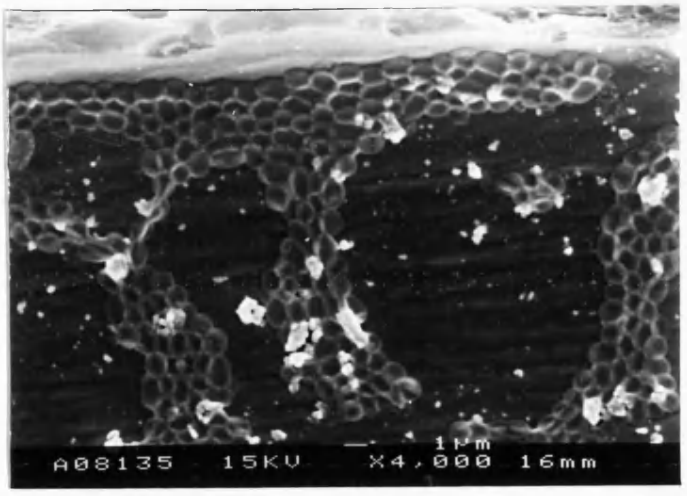

Figure 5.15 A cryo-fixed sample of Picabiol with 3 day biofilm of *K. pneumoniae* growing between the side walls of the secondary xylem vessels of the carbon.

After 7 days culture, growth on the Picabiol was more abundant (Figure 5.16), and there was generally more growth over the carbon surface. Figure 5.16 shows the transverse aspect of the xylem vessels and depicts the honeycomb nature of the carbon. Each vessel is approximately 30 µm in diameter. Again bacteria have colonized the sheltered areas, the delicate exopolysaccharide, seemingly supporting growth of cells, may be seen plugging the open ended vessels. The thinness of this layer is manifest from the holes within it, possibly caused during sublimation which may have partially dehydrated the exopolysaccharide. Interestingly where the biofilm

is thicker, at the confluence of the wood cells, little exopolysaccharide is in evidence, possibly because the bacterial cells needed no additional support.

Figure 5.17 is an enlargement of the previous figure, centered on the confluence of the pore walls. Individual cells can be seen clearly where they have built up into a thick mass in the sheltered regions. The smoothness of the cylinders of Picabiol may be compared with the roughness of F400 at a similar magnification (see Figure 5.18).

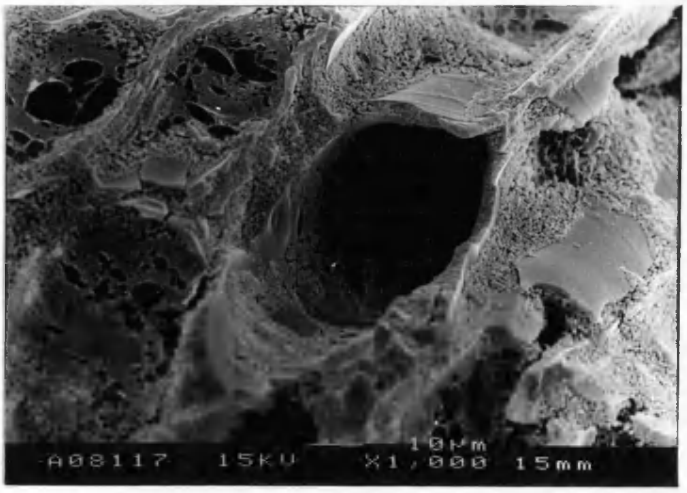

Figure 5.16 Cryo-fixed sample of Picabiol showing the carbon structure in transverse section through the wood vessels. Cells of *K. pneumoniae* are colonizing the carbon surface.

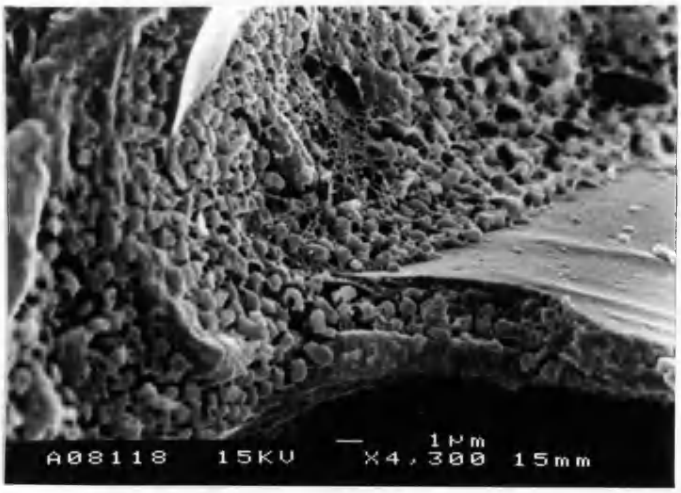

Figure 5.17 A portion of the previous figure enlarged to picture the area of thick *K. pneumoniae* growth.

5.3.2.2 F400

The small rounded cells of 3 day *K. pneumoniae* nestled between the surface protrusions and other matter on the surface of F400 are shown in Figure 5.18. Cells of the 3 day culture have successfully attached to the irregular carbon surface, but there

has been little proliferation. Lower magnification shows that the cells can proliferate and colonize the carbon, but the surface area available seems to be limited to the channels of diameter 10-3 μm in width, with little growth upon the prominent exposed regions.

Following a 7 day incubation with F400 the production of biofilm was enhanced (Figure 5.19), and some areas of densely packed cells, swathed with exopolysaccharide were found on the carbon.

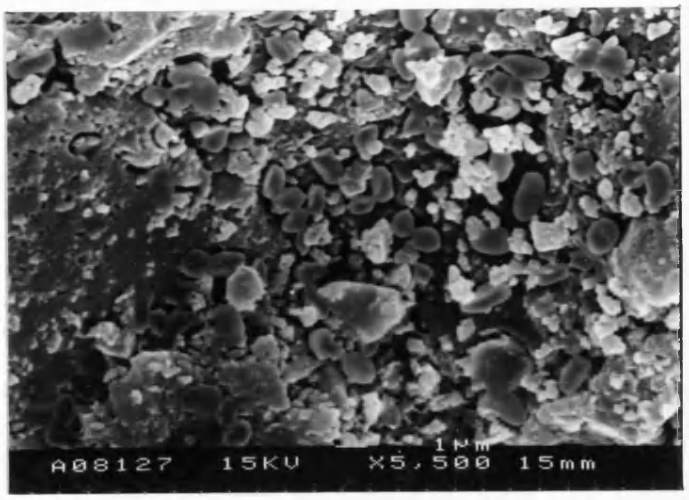

Figure 5.18 Cryo-fixed cells of *Klebsiella pneumoniae* upon the surface of carbon F400.

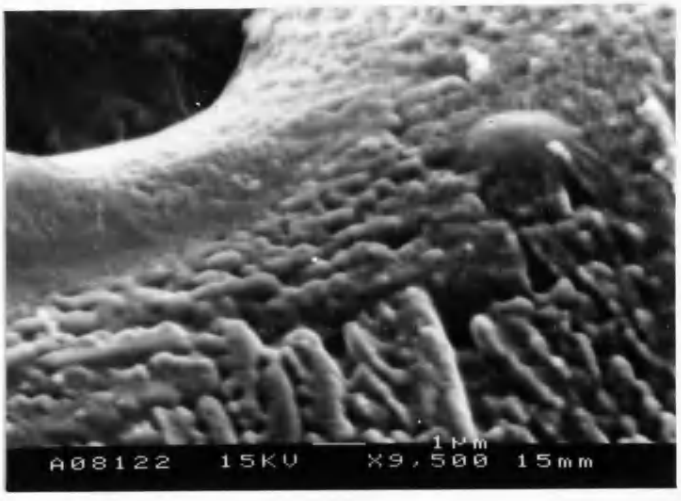

Figure 5.19 A high magnification micrograph of 7 day *K. pneumoniae* biofilm over the surface of F400. Cryo-fixed and viewed on the cold stage at -180°C.

Following a qualitative assessment of much of the carbon surface, it was considered that colonization of F400 was sparse in comparison with Picabiol.

5.4 Carbon isotherm studies

5.4.1 Single metal systems

The Figures 5.20-5.25 show the Freundlich isotherm plots of single metal sorption (Cd(II), Cr(VI), Ni(II)) by the two carbons Filtrasorb 400 (F400) and Picabiol. Data was gained from batch equilibrium studies where 0.5 g carbon was challenged with metal solutions of concentrations between 5 and 100 mg/l. Each plot compares adsorption by a carbon and attached biofilm, with those by carbon wetted with either water or tryptone, and without biofilm. The non-activated carbon (anthracite) isotherms are not displayed here since metal loading even with biofilm at the highest concentrations was negligible; anthracite did not accumulate metal from solution.

The isotherm shows the distribution, at constant temperature, of target substances between the adsorbed phase and bulk solution at equilibrium. It is a plot of the amount of impurity (in this case metal) adsorbed per unit weight of carbon (in milligrams metal per gram), versus the liquid phase equilibrium metal ion concentration (expressed as milligrams metal per litre sorbate). Standard deviations for the data points have not been represented graphically since the relatively small errors become negligible when presented on a logarithmic scale. Errors were always within 10%, and more usually less than 6%. The largest standard deviation about any one point of each plot is quoted in the figure legend.

5.4.1.1 Freundlich isotherm constants

The isotherm constants of the plots were derived by linear regression on each data set in the form of the Freundlich isotherm:

$$\frac{x}{m} = KC_e^{1/n}$$

where: $\frac{x}{m}$ = loading (mass of solute adsorbed per unit mass of adsorbent) (mg/g),

C_e = equilibrium concentration of the solute after adsorption (mg/l),

K and $1/n$ are constants characteristic of the system.

The Freundlich constants for each of the experimental conditions are presented in Table 5.2.

Table 5.2 Freundlich isotherm constants for single metal batch equilibrium systems.

Carbon	Metal	Condition	K	1/n	Correlation coefficient (R^2)
Picabiol	Cd	water	2.36	0.18	0.95
		tryptone	3.98	0.18	0.91
		biofilm	10.11	0.30	0.90
	Cr	water	21.66	0.35	0.95
		tryptone	0.83	1.16	0.99
		biofilm	0.0004	2.90	0.95
	Ni	water	1.75	0.20	0.94
		tryptone	2.88	0.24	0.94
		biofilm	5.32	0.27	0.89
F400	Cd	water	0.39	0.18	0.21
		tryptone	2.47	0.15	0.75
		biofilm	4.28	0.18	0.74
	Cr	water	15.99	0.93	0.99
		tryptone	0.17	1.82	0.99
		biofilm	0.001	3.16	0.99
	Ni	water	0.34	0.39	0.85
		tryptone	2.18	0.16	0.71
		biofilm	2.85	0.17	0.63

The metal uptake isotherms are presented on the basis of the mass of metal accumulated per gram of sorbent. The mass basis is important when quantifying the metal capacity in terms appropriate to industrial applications, where the metal loading of a waste-water generally is expressed in ppm or mg/l.

Measurement of concentration by molarity rather than mass, however can be a useful tool, and is more appropriate when investigating competition in multi-component metal solutions. Comparison of metal uptakes on a molar basis will indicate the total number of metal ions adsorbed (as opposed to total *mass*) and may give some estimate of the total number of sites available to each metal on the sorbent matrix.

5.4.1.2 Nickel

The three isotherms of divalent nickel uptake by plain and modified F400 are displayed in Figure 5.20. The two parallel isotherms of tryptone wetted F400, with and without biofilm, lie above that of plain F400 wetted with sterile distilled water alone. Biofilm coated carbon, the higher of the two parallel isotherms; adsorbed the greatest load of nickel per gram of carbon over the range of equilibrium concentrations, 0.6-38 mg/l. Plain carbon demonstrated the least of the nickel loadings at equilibrium concentrations of between 4.7 and 100 mg/l. Thus *K. pneumoniae* biofilm enhanced the capacity of F400 for divalent nickel, as did the presence of tryptone in the wetting solution. Plain F400 wetted with water only, was the least efficient at concentrating the nickel at its surface.

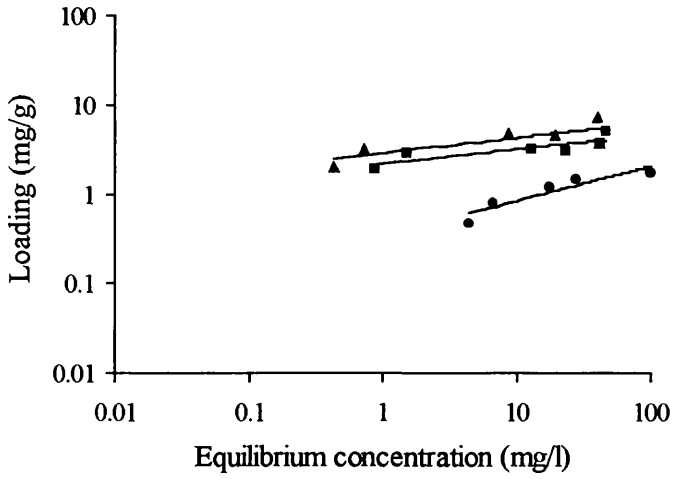

Figure 5.20 Freundlich isotherms for nickel sorption by F400: without biofilm and wetted in; water (•), tryptone (▪) and; with biofilm cultured in tryptone (▲) [SD ± 4%].

The greater part of the enhancement in metal uptake capacity was conferred not by the activity of the biofilm but by the presence of tryptone in the system during the wetting process, demonstrating the importance of the tryptone control. At an

equilibrium concentration of 10 mg/l, for example, the nickel loadings of F400 were: water control, 0.8 mg/g; tryptone control, 3.1 mg/g; biofilm coated, 4.2 mg/g carbon; i.e. 68% of the 3.4 mg increase in loading was attributable to changes in the system effected by tryptone, and not the cells which contributed only 32% of the enhancement. This difference between the tryptone control and uptake modulated by the biofilm system, although seemingly small when plotted using a logarithmic scale, was indeed significant at the 5% probability level when regression lines were compared by analysis of variance.

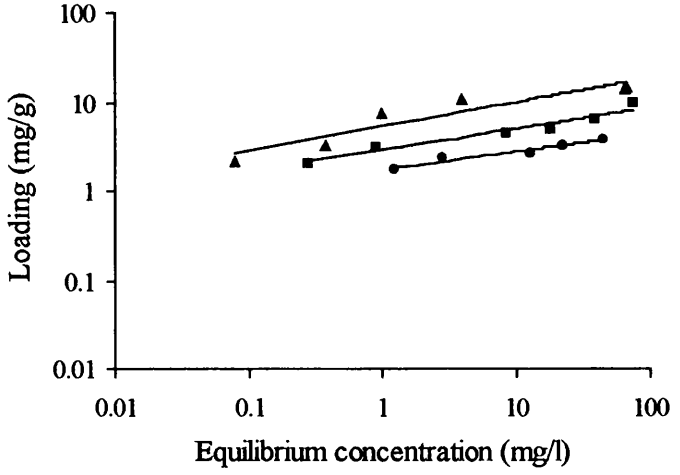

Figure 5.21 Freundlich isotherms for nickel sorption by Picabiol: without biofilm and wetted in; water (•), tryptone (▪) and; with biofilm cultured in tryptone (▲) [SD ± 4%].

Nickel sorption by Picabiol is plotted in Figure 5.21. The same ranking is exhibited as nickel adsorption by F400, although the Picabiol nickel capacity was higher than that of F400 under parallel conditions. Loadings, for example at 10 mg/l equilibrium concentration, were: water control, 2.8 mg/g; tryptone control, 5.0 mg/g; and biofilm coated carbon, 9.9 mg/g carbon. The enhancement conferred by the presence of tryptone in the Picabiol system was less marked than was the case with F400 (at an equilibrium concentration of 10 mg/l, F400 loading in the presence of tryptone was 3.7 times greater than in its absence but for Picabiol this increase was less than 2-fold). Contrastingly the positive effect of the biofilm was greater when on Picabiol (at 10 mg/l loading was twice that of carbon without biofilm, where in the case of F400 enhancement was only by 34%).

5.4.1.3 Cadmium

Adsorption isotherms for divalent cadmium uptake by F400 and Picabiol are shown in Figures 5.22 and 5.23 respectively. The cadmium isotherms strongly reflect those encountered in adsorption of nickel by F400 and Picabiol. The ranking of carbon was the same as that for adsorption of nickel; for both F400 and Picabiol, biofilm coated carbon was more efficient than carbon without biofilm, and tryptone always lead to a greater loading than water wetted carbon, over the range of metal concentrations of these experiments.

The F400 isotherms for cadmium sorption (Figure 5.22) are parallel (and thus have the same 1/n term, see Table 5.2). The relative efficiencies, over the range of experimental concentrations of the parallel isotherms of biofilm coated and water wetted carbon, remains constant. At 10 mg/l cadmium concentration loadings were: water control, 0.6 mg/g; tryptone control, 3.5 mg/g and biofilm coated 6.4 mg/g. The presence of tryptone increased the metal loading, over that of the plain water system, by 6-fold, biofilm further increased the loading by almost 2-fold.

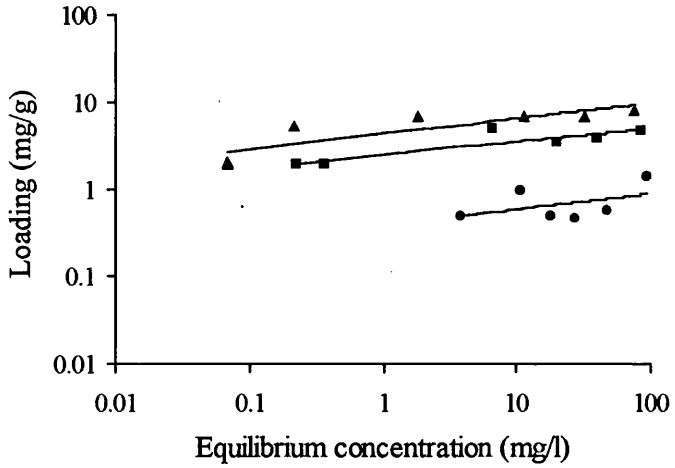

Figure 5.22 Freundlich isotherms for cadmium sorption by F400: without biofilm and wetted in; water (•), tryptone (▪) and; with biofilm cultured in tryptone (▲) [SD ± 9%].

When compared with the capacity of F400 for nickel (on a mass basis), the same mass of each metal was adsorbed when F400 was wetted with tryptone. More cadmium than nickel was accumulated when biofilm was present, and more nickel was accumulated than cadmium by virgin F400.

The Freundlich isotherms for cadmium adsorption by Picabiol (Figure 5.23) again show the same order of uptake efficiency. In keeping with nickel uptake by Picabiol, the addition of tryptone did not confer such an advantage as did the addition of a *K. pneumoniae* biofilm. This enhancement of metal loading in the presence of biofilm is the most significant of the carbon biofilm systems so far discussed, and the gradient of the isotherm shows that the biofilm presence becomes yet more valuable, in terms of mass of metal taken up, (over and above the control conditions) as the equilibrium concentration increased. At 40 mg/l equilibrium concentration, biofilm loading was 7 times more than that of the water control, whereas at 1 mg/l, loading in the presence of the biofilm was only 4-fold that of the water control.

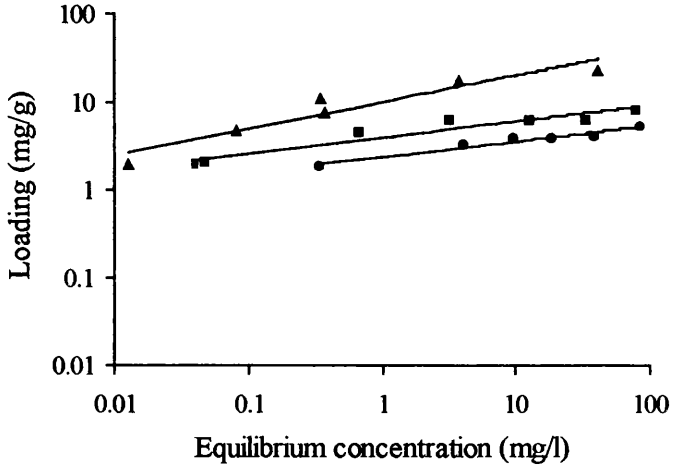

Figure 5.23 Freundlich isotherms for cadmium sorption by Picabiol: without biofilm wetted in; water (•), tryptone (▪) and; with biofilm cultured in tryptone (▲) [SD ± 4%].

Picabiol was a better adsorber of cadmium than F400 under the same conditions.

5.4.1.4 Chromium

Isotherms (Figures 5.24 and 5.25) show that the ranking of metal loading on carbon under different conditions is reversed in the single component chromium system (at concentrations between 0.1 mg/l and 40 mg/l for F400, and 0.01 and 40 mg/l for Picabiol). It is clear that biofilm confers no advantage regarding metal uptake, and only serves to reduce the carbon's capacity for chromium. The same is true for carbon wetted with tryptone rather than water.

The slope of the chromium isotherms in contrast to those of nickel and cadmium is very steep, indicating a very large increase in metal loading on the carbon for small increments in the equilibrium concentration.

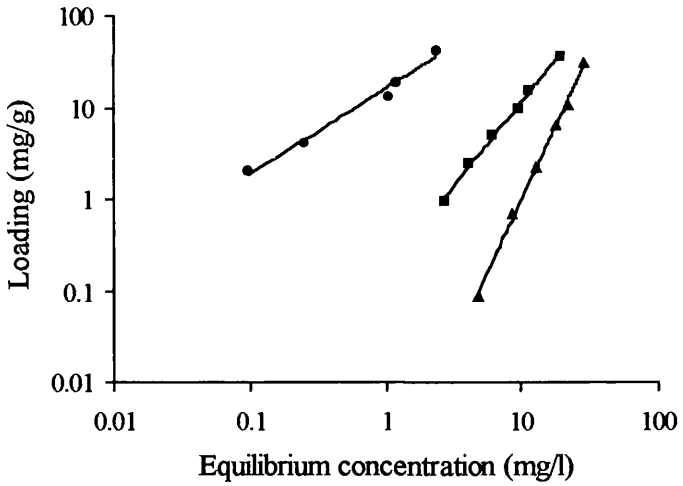

Figure 5.24 Freundlich isotherms for chromium sorption by F400: without biofilm wetted in; water (•), tryptone (▪) and; with biofilm cultured in tryptone (▲) [SD ± 4%].

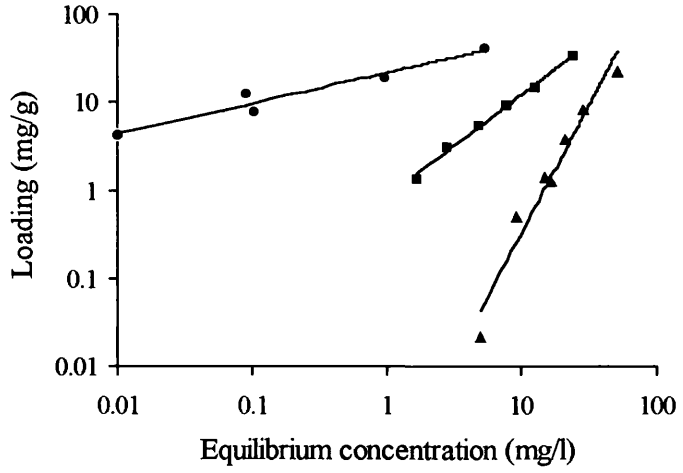

Figure 5.25 Freundlich isotherms for chromium sorption by Picabiol: without biofilm wetted in: water (•), tryptone (▪) and; with biofilm wetted in tryptone (▲) [SD ± 5%].

Virgin Picabiol demonstrates the greatest promise for chromium adsorption, and achieved the highest loading per gram of carbon of any of the test metals below an equilibrium concentration of 2 mg/l. At concentrations above this, only virgin F400 achieved a higher loading.

The shallow gradients of most of the isotherms encountered during these metal uptake studies lend the carbons to a batch operating system, in that the carbon loading is maintained even as the concentration of the metal solution decreases. However, for column operation (favoured by industry since it is a continuous process and hence cheaper to operate) isotherms with a steeper gradient are acceptable so long as the loading is high at the influent concentration (the conditions at which adsorption takes place in the mass transfer zone).

5.4.1.5 Metal loadings

The single metal loadings of the two activated carbons at equilibrium concentrations of 10 mg/l and 0.1 mg/l are shown in Tables 5.3 and 5.4 respectively. The metal loadings were calculated from the Freundlich isotherm constants of Table 5.2.

At 10 mg/l equilibrium concentration (Table 5.3) the loadings of Cr in particular are impressive, yet these are pertinent only when gross contamination has led to a very concentrated waste stream. In general the metal loading of Picabiol at 10 mg/l was superior to that of F400, although Cr was better accumulated by F400, particularly in the virgin state. Nickel and Cd loadings were relatively low and improved in the presence of biofilm.

Table 5.3 Apparent metal loading at 10 mg/l equilibrium concentration.

Metal	Carbon	Apparent loading (mg metal/g carbon)		
		water	tryptone	biofilm
Nickel	F400	0.8	3.1	4.2
	Picabiol	2.8	5.0	9.9
Cadmium	F400	0.6	3.5	6.5
	Picabiol	3.6	6.1	20.3
Chromium	F400	137.7	11.3	1.5
	Picabiol	48.6	12.1	0.3

The metal loadings at the lower equilibrium concentration of 0.1 mg/l (Table 5.4) are of great interest since many of the industrial rinsewaters may be dilute, and discharge limits set by regulating authorities very low because of the large volumes of water that are returned to the water supply.

Table 5.4 Apparent metal loading at 0.1 mg/l equilibrium concentration.

Metal	Carbon	Apparent loading (mg metal/g carbon)		
		water	tryptone	biofilm
Nickel	F400	0.1	1.5	1.9
	Picabiol	1.1	1.7	2.8
Cadmium	F400	0.3	1.8	2.8
	Picabiol	1.6	2.6	5.0
Chromium	F400	1.9	0.0	0.0
	Picabiol	9.7	0.1	0.0

Relative to the 10 mg/l equilibrium concentration the metal loadings at 0.1 mg/l remained high, fitting the carbons for use in polishing dilute wastes. Again Picabiol metal loadings were superior to those of F400 in all cases except Cr adsorption by biofilm covered carbon. Chromium loading of both carbons in the presence of biofilm was negligible.

5.4.2 Mixed metal systems

Carbons were challenged with equal concentrations of the three metals Cr, Cd and Ni in a ternary metal solution. Two starting concentrations; 20 mg/l and 50 mg/l, were used to provide a comparison between individual metal uptakes from single component solutions plotted as Freundlich isotherms (Section 5.4.1), and each of the metals from the multi-component system plotted on the isotherm as points. The loading of each metal from the mixed solution was plotted against its own equilibrium metal concentration.

5.4.2.1 Nickel

Nickel uptakes by F400 and Picabiol from the ternary metal solutions (Cd, Cr, and Ni) are depicted in Figures 5.26 and 5.27 respectively. Generally Ni uptake from a mixed metal solution by F400 (Figure 5.26) was best achieved by biofilm covered carbon. Nickel uptake was depressed by the presence of other metals under all conditions; whether water or tryptone wetted, or with an attached biofilm.

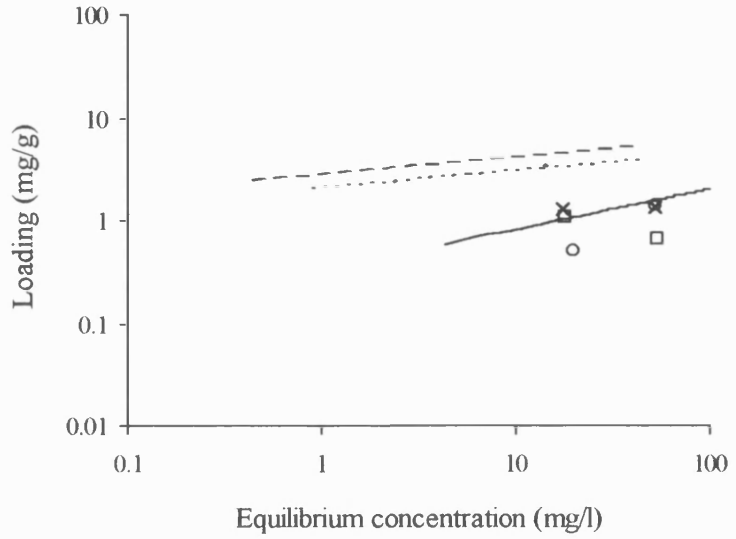

Figure 5.26 Freundlich isotherms of nickel sorption by F400 from single metal (lines) and mixed metal systems (symbols): without biofilm and wetted in; water (—) (○), tryptone (- - -) (□) and; with biofilm (– – –) (×), [SD for mixed metal data ± 8%].

When wetted with water, Ni uptake from the mixed solution, and thus its loading on the carbon, increased with increasing Ni concentration. This was not the case in the presence of biofilm where Ni loading did not increase with increased starting or equilibrium concentrations. Under the conditions of tryptone wetting but without subsequent biofilm development there was a decrease in Ni loading as its equilibrium concentration increased. This is an unusual phenomenon, and indicates that a more complex system than simple metal sorption prevails. It may be that components of the tryptone medium which were pre-adsorbed on to the carbon and the other metals in the mixed metal solution together have a significant negative effect upon the Ni loading, as the metal concentration is increased. Further to this, the presence of biofilm moderated negative loading.

The nickel capacity of Picabiol is shown in Figure 5.27. Again the points plotted are below those of the corresponding single metal system; the Ni capacity of Picabiol was reduced when part of a multi-component system. The effect was less marked than that demonstrated by F400. The loading of Ni on water and tryptone wetted carbons increased with increasing equilibrium concentration, but decreased when a biofilm was present on the carbon. Nickel removal from the mixed metal solution was best achieved when a biofilm was present over the carbon. The suppression of Ni loading by other metals was least after wetting in water and at the higher equilibrium concentration.

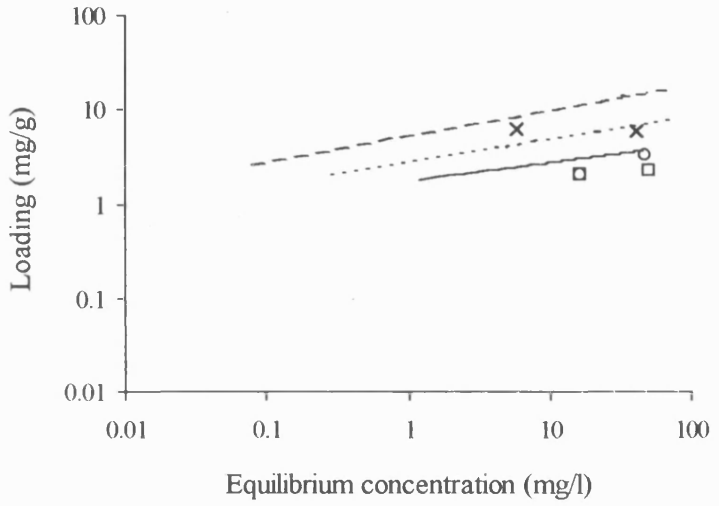

Figure 5.27 Freundlich isotherms of nickel sorption by Picabiol from single metal (lines) and mixed metal systems (symbols): without biofilm and wetted in; water (—) (○), tryptone (- - -) (□) and; with biofilm (– – –) (×), [SD for mixed metal data ± 7%].

5.4.2.2 Cadmium

Cadmium adsorption isotherms and cadmium uptake data from mixed metal solutions are presented in Figures 5.28 and 5.29. The presence of other metals significantly reduced the loading of cadmium on F400 when tryptone and biofilm had been in contact with the carbon. At the higher concentration only the tryptone trial suffered from a loading less than at the lower concentration. In the presence of the biofilm this was not the case; either the phenomenon did not occur or the biofilm masked its incidence through its own cadmium sorption capacity. The highest cadmium loading

from the mixed metal solution was achieved by biofilm coated F400. Cadmium uptake on to virgin (water wetted) F400 was enhanced by the presence of Cr and Ni.

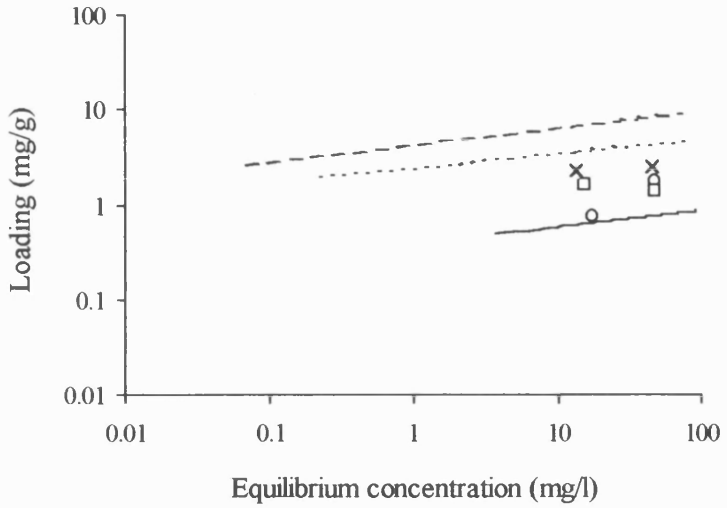

Figure 5.28 Freundlich isotherms of cadmium sorption by F400 from single metal (lines) and mixed metal systems (symbols): without biofilm and wetted in; water (—) (○), tryptone (- - -) (□) and; with biofilm (– – –) (×), [SD for mixed metal data ± 10%].

Cadmium sorption by Picabiol is shown in Figure 5.29. Under all conditions the Cd loading from mixed solution was less than that from a solution of Cd alone, when comparing Cd (rather than total metal) at the same equilibrium concentration. At the higher of the two equilibrium concentrations the same loading was achieved independent of the presence of tryptone or biofilm; all three data points coincided. At the lower equilibrium concentration the biofilm enhanced Cd loading. The biofilm coated carbon described a negative loading, although tryptone did not.

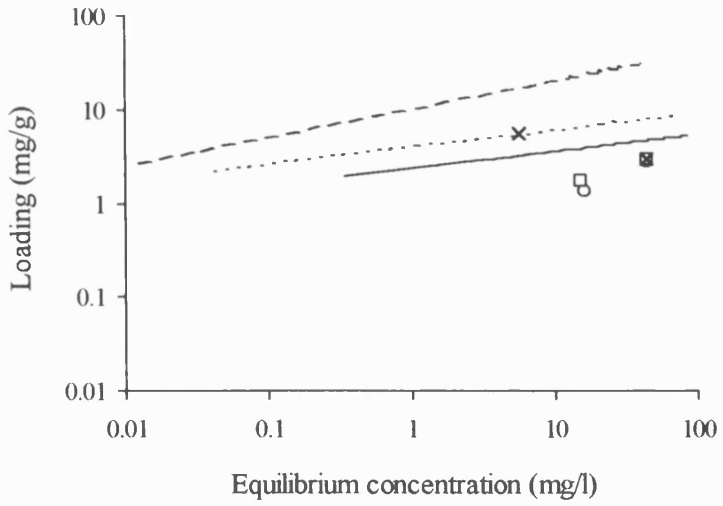

Figure 5.29 Freundlich isotherms of cadmium sorption by Picabiol from single metal (lines) and mixed metal systems (symbols): without biofilm and wetted in; water (—) (○), tryptone (- - -) (□) and; with biofilm (– – –) (×), [SD for mixed metal data ± 10%].

5.4.2.3 Chromium

Chromium isotherms and data points for Cr uptake from mixed metal solutions by F400 and Picabiol are presented in Figures 5.30 and 5.31 respectively. Under all incubation conditions the F400 loading (Figure 5.30) increased with increasing chromium equilibrium concentration. The loading increased markedly with concentration. Chromium loading in a water system remained largely unaffected by the presence of the other metals, implying that under such conditions Cd(II) and Ni(II) ions may not compete for the same active sites as the chromium. The loading of Cr in both tryptone and biofilm systems was enhanced significantly by the presence of the other metals, however the highest chromium loading, even at low equilibrium concentrations, was achieved by virgin (water wetted) F400.

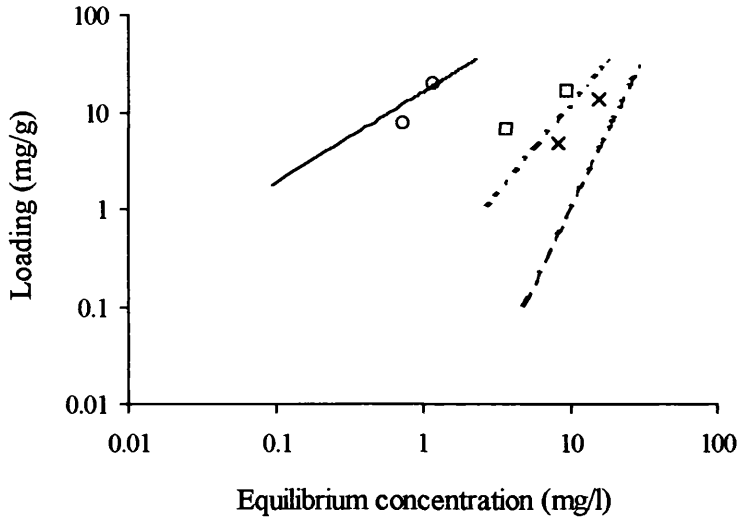

Figure 5.30 Freundlich isotherms of chromium sorption by F400 from single metal (lines) and mixed metal systems (symbols): without biofilm and wetted in; water (—) (o), tryptone (- - -) (□) and; with biofilm (– – –) (x), [SD for mixed metal data ± 4%].

The chromium loading of Picabiol under different incubation conditions is shown in Figure 5.31. Increasing the equilibrium concentration always increased the loading of Cr on the Picabiol. The greatest Cr loading from the mixed solution was achieved by virgin carbon. The Cr loading from mixed metal solution on virgin Picabiol was depressed at the lower of the two equilibrium concentrations when compared to the single metal isotherm, however at the higher concentration the loading from mixed solution was close to coinciding with that predicted by the single metal isotherm. At the lower equilibrium concentrations under the conditions of tryptone and biofilm presence, the loading of Cr from mixed solution was significantly more than was the case from single metal solutions at the same equilibrium concentration. At higher concentrations this effect was less marked although the presence of Cd and Ni still enhanced the Cr loading.

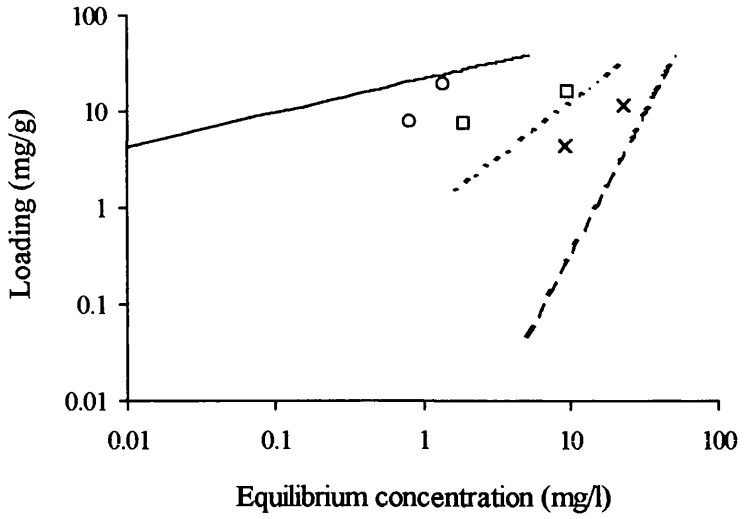

Figure 5.31 Freundlich isotherms of chromium sorption by Picabiol from single metal (lines) and mixed metal systems (symbols): without biofilm and wetted in; water (—) (o), tryptone (- - -) (□) and; with biofilm (– – –) (×), [SD for mixed metal data ± 4%].

5.4.3 Molar basis mixed metal isotherms

Comparing metal loadings on carbon by a mass basis is a useful technique, particularly for assessing the suitability of a carbon for use in an industrial setting. In such cases the metal waste to be dealt with would be quantified by mass, for example in mg/l or ppm, and a comparison of carbon capacities made by the mass of metal that could be taken up by each gram, kilogram or tonne of carbon.

In a research setting, when mixed metal solutions in particular are studied, a molar basis for presenting isotherms may be more useful in discerning what competition, if any, occurs in the system. A molar basis can be used to calculate the *total number* of metal ions in solution (by use of Avogadro's constant), which can be related more directly to the total number of active sites which are available on the carbon surface.

Molar masses of the three metals are; Cd (112.4 g), Ni (58.71 g), Cr (51.996 g), hence the same mass of cadmium in solution would contain approximately half the number of ions as a nickel or chromium solution. To plot the isotherms on a molar basis allows the comparison of uptakes under conditions of ion concentration that are directly comparable. The isotherms from Section 5.1.1 have been replotted on a molar

basis in Figures 5.32-5.37. The three metals were compared under the same conditions, i.e. Figures 5.32 and 5.33 describe the uptake of Cd, Ni and Cr by virgin carbons, Figures 5.34 and 5.35 show the metal loadings for the tryptone control, and Figures 5.36 and 5.37 compare metal uptakes by biofilm covered Picabiol and F400 respectively. Individual metal loadings from ternary metal solutions are plotted on the same graphs as points.

5.4.3.1 Water

Comparing the single metal isotherms for virgin Picabiol uptakes (Figure 5.32) on a molar basis it was clear that all concentrations the molar loading of chromium was superior to Ni and Cd. The isotherms for single metal Ni and Cd uptakes are parallel, demonstrating that the relative efficiency of removal of these metals by Picabiol remained constant but with a higher Ni loading than Cd, in contrast to the mass basis isotherms where Cd loading was always slightly above that of Ni.

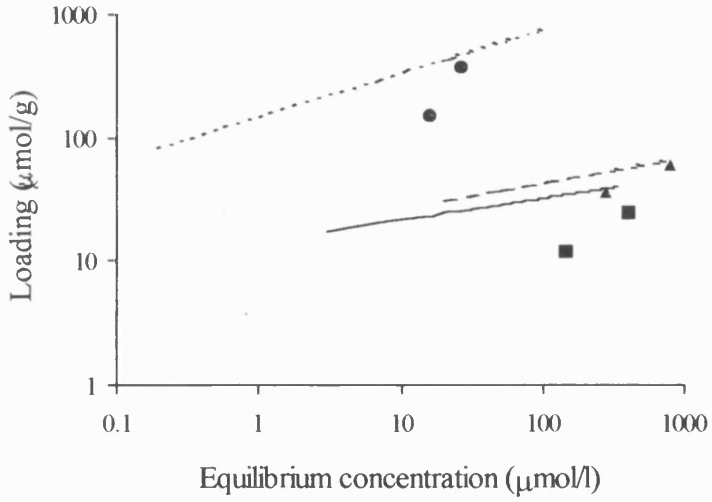

Figure 5.32 The molar basis Freundlich isotherms for metal sorption by water wetted Picabiol carbon from: single component systems (lines) and each component from a mixed system (symbols); Cd (—) (■), Ni (– – –) (▲), Cr (- - -) (●), [SD mixed metal data ± 4%]..

All of the points representing the metal loadings from the ternary systems were found below their respective single metal isotherms showing that the presence of other metals reduced the loading of Cr, Cd and Ni. On virgin Picabiol the Cd loading

suffered a greater reduction than did the Ni. The loading of Ni from mixed metal systems was greater than that of Cd when plotted on either a mass or a molar basis. Chromium was subject to a greater reduction in loading at the lower equilibrium concentration.

The single metal isotherms for uptake by virgin F400 (Figure 5.33) show that Cr loading on a molar basis, was superior to the loading of either Ni or Cd, and Ni loading was greater than that of Cd, in accord with the mass basis isotherms.

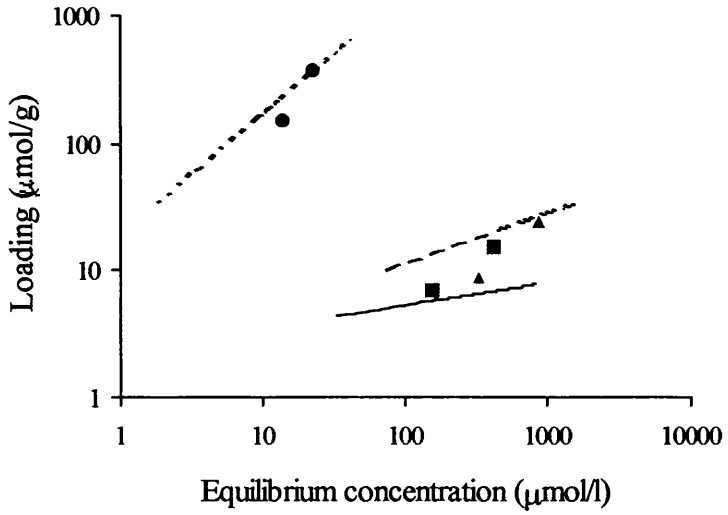

Figure 5.33 The molar basis Freundlich isotherms for metal sorption by water wetted F400 carbon from: single component systems (lines) and each component from a mixed system (symbols); Cd (—) (■), Ni (– – –) (▲), Cr (- - -) (●), [SD mixed metal data ± 4%].

Chromium and Ni loadings on virgin F400 were compromised in the presence of other metals, at the lower of the two concentrations, whilst Cd loading was enhanced. Unlike Picabiol, where all the metals from the mixed system displayed a reduced loading when compared to the single component system, the F400 loadings were more varied. At the lower of the two equilibrium concentrations, loading of Cr from the ternary metal solution was less than that predicted by the single metal isotherm, whereas at the higher concentration the point lies on the isotherm. The effect of the reduction in Ni loading in the presence of other metals was large at the lower equilibrium concentration but less marked at the higher concentration, i.e. at an

equilibrium concentration of 332 μmol/l Ni loading from the single component solution was 18.3 μmol/g, and from the mixed solution was 8.7 μmol/g, at the higher concentration of 882 μmol/l the loadings were 26.8 μmol/g (single) and 23.9 μmol/g (mixed). Cadmium loading was enhanced in the presence of other metals and more so at the higher equilibrium concentration.

5.4.3.2 Tryptone

The molar isotherms of single component metal systems and ternary metal data points for carbons under tryptone wetting conditions, are presented for Picabiol and F400 in Figures 5.34 and 5.35 respectively.

Plotting the single metal isotherms of Picabiol adsorption for the tryptone control (Figure 5.34) on a molar basis rather than mass basis demonstrated that the carbon affinity was greater for Ni(II) ions than for Cd(II) ions. This would not be clear from a mass based isotherm as the *mass* of Cd sorbed by the carbon was greater than that of Ni, although this is due only to the much larger molar mass of Cd. Chromium loading from the single metal system was only greater than the Ni and Cd systems at an equilibrium concentration greater than 70 and 50 μmol/l respectively.

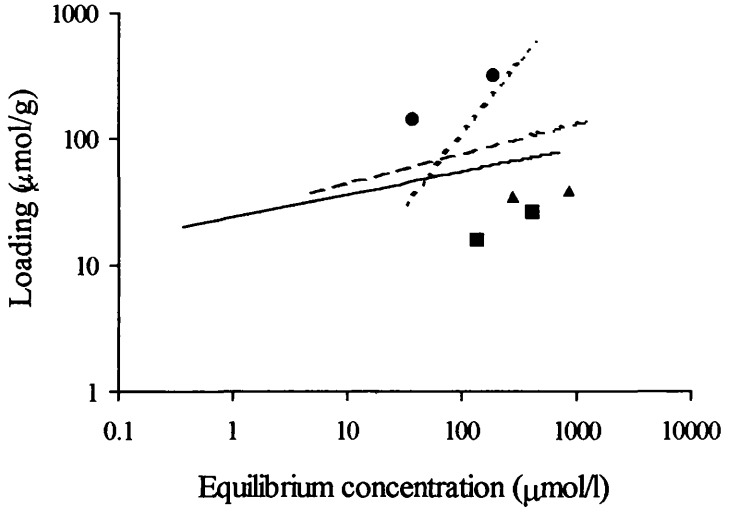

Figure 5.34 The molar basis Freundlich isotherms for metal sorption by tryptone wetted Picabiol carbon from: single component systems (lines) and each component from a mixed system (symbols); Cd (—) (■), Ni (– – –) (▲), Cr (· · ·) (●), [SD ±7%].

The Cr loading of Picabiol (Figure 5.34) from a mixed solution was enhanced by the presence of other metals, this effect was more significant at the lower equilibrium concentration. Nickel and cadmium ion uptakes were both suppressed when in the ternary mix. The molar plot demonstrated that the Ni loading from a mix was greater than Cd sorption for Picabiol wetted with tryptone, whereas when plotted by mass the greater affinity for Ni was again masked due to the high molar mass of Cd.

Figure 5.35 is a plot of single molar metal isotherms of the tryptone control of F400, with the additional points of metal uptake from the ternary metals solutions. When plotted on a mass basis the Cd and Ni single metal isotherms were coincident, however by molarity it is clear that the tryptone wetted F400 demonstrates a greater loading of Ni ions than Cd ions. The isotherms are parallel; their relative uptake efficiency across the range of concentrations therefore remains constant. Chromium loading on to tryptone wetted F400 only exceeded loadings of Cd and Ni at equilibrium concentrations above 62 and 82 μmol/l respectively.

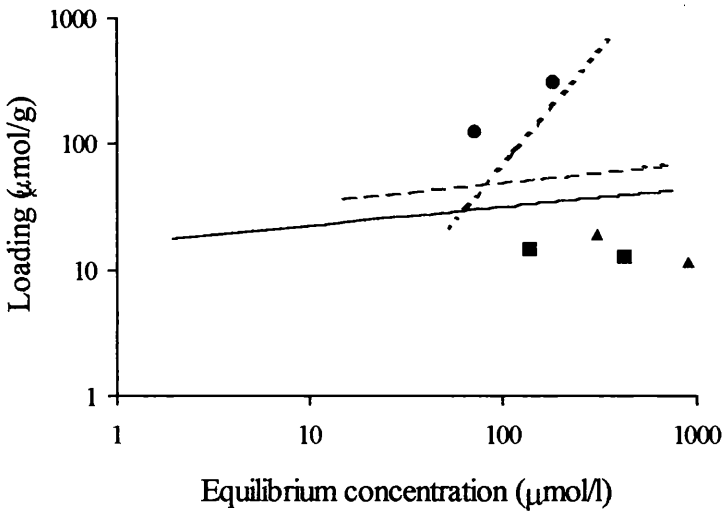

Figure 5.35 The molar basis Freundlich isotherms for metal sorption by tryptone wetted F400 carbon from: single component systems (lines) and each component from a mixed system (symbols); Cd (—) (■), Ni (– – –) (▲), Cr (- - -) (●), [SD ± 10%].

Chromium loading from the mixed metal solutions was enhanced in the presence of other metals, and more so at the lower equilibrium concentrations. The loadings of Cd and Ni from the mixed systems demonstrated a loading lower than that predicted by

the single metal isotherms, and upon linking each of the pairs of data points a negative gradient became evident. This is a phenomenon shared by both Ni and Cd but peculiar to carbon F400 after wetting with tryptone (also found with biofilm covered Picabiol, Figure 5.36), and not found when biofilm was grown on the carbon (see Figure 5.37). The reduction in loading experienced by Cd and Ni became increasingly marked as the equilibrium concentration of these metals increased. At the experimental equilibrium concentrations the Ni ion loading was slightly greater than the Cd loading, whereas when loadings were measured by mass, this case was reversed.

5.4.3.3 Biofilm

The molar isotherms of single component metal systems and ternary metal data points for carbon with biofilm are presented for Picabiol and F400 in Figures 5.36 and 5.37 respectively.

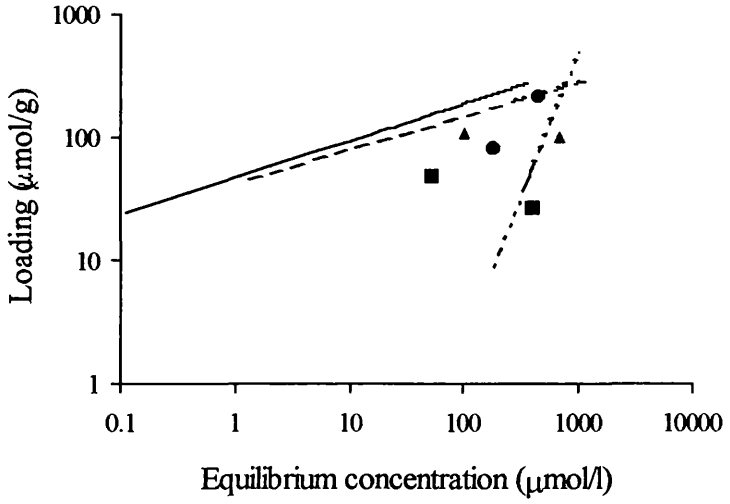

Figure 5.36 The molar basis Freundlich isotherms for metal sorption by biofilm covered Picabiol carbon from: single component systems (lines) and each component from a mixed system (symbols); Cd (—) (■), Ni (– – –) (▲), Cr (- - -) (●), [SD ± 6%].

Single system Ni and Cd loadings on Picabiol (Figure 5.36) were superior to those of Cr unless in excess of 730 µmol/l equilibrium concentration in the case of Ni, and by extrapolation, 890 µmol/l for Cd. In agreement with the mass data, Cd loadings for single component systems were higher than those of Ni, although at very low equilibrium concentrations i.e. < 1 µmol/l these would be expected to converge. The

loading of chromium ions from mixed metal solution onto Picabiol with biofilm was enhanced by the presence of the other metals, the loadings of Cd and Ni, in contrast, were much reduced, particularly as the equilibrium concentration increased. Cadmium loadings were compromised to a greater extent than Ni. A similar phenomenon of negative loading of Ni and Cd occurred for biofilm coated Picabiol sorption from mixed metal solution as occurred with tryptone wetted F400 (Figure 5.35).

When plotted on a molar basis the single metal isotherms of F400 with attached biofilm (Figure 5.37) demonstrated an altered ranking when compared with those expressed as mass loadings. The cases of Cd and Ni are interesting, the isotherms are parallel when plotted by either a mass or molar basis, however when considering metal masses the Cd had significantly higher loading than Ni (at P 0.05). Plotted by molarity this trend was reversed; the number of nickel ions adsorbed on to the carbon/biofilm surface was slightly higher than the number of Cd ions adsorbed, although the parallel isotherms almost coincide. Chromium loading was higher than that of Cd and Ni only at equilibrium concentrations in excess of 300 μmol/l.

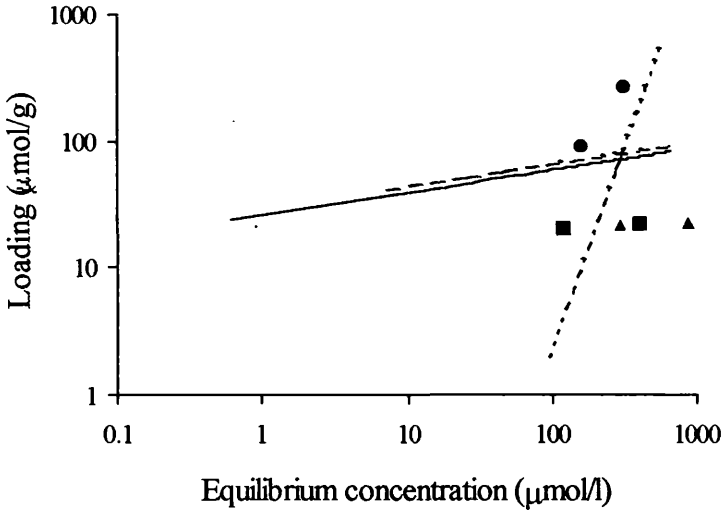

Figure 5.37 The molar basis Freundlich isotherms for metal sorption by biofilm covered F400 carbon from: single component systems (lines) and each component from a mixed system (symbols); Cd (—) (■), Ni (– – –) (▲), Cr (- - -) (●), [SD ± 7%].

At the concentrations considered in these experiments the molar Cr loading from ternary solution was enhanced by the presence of the other metals. The loadings of Cd

and Ni were, in contrast, reduced. The Cr uptake from solution increased at the expense of Cd and Ni loading.

Interestingly a line drawn through the Cd points (uptake from ternary solution) also took in those of Ni. The gradient of this line was almost zero, there was no increase of the loading as the number of ions in equilibrium solution increased. This suggests not only that the carbon surface was saturated i.e. even though there are increasingly more ions available there are no active sites left on the carbon/biofilm which are open to the Cd and Ni, but also that these metals possibly compete for the same sites. The coincidental isotherms and data points, when plotted by molarity suggest that the site and mode of Ni and Cd adsorption are the same. This information only becomes apparent when data is plotted on a molar basis since the masses erroneously suggest that twice the amount of Cd compared to Ni was accumulated by the system, so appearing to be preferentially adsorbed.

5.4.4 Spent media effects

The effect of spent tryptone medium upon Cr and Cd removal from solution by F400 and Picabiol is shown in Figures 5.38 and 5.39 respectively. *Klebsiella pneumoniae* was cultured in 200 ml of tryptone and following incubation the spent tryptone was harvested by centrifugation and filtration. Spent tryptone was added to batch equilibrium flasks of 0.5 g carbon as before. The metal loading in the presence of spent tryptone was compared with that of fresh tryptone and biofilm on carbon.

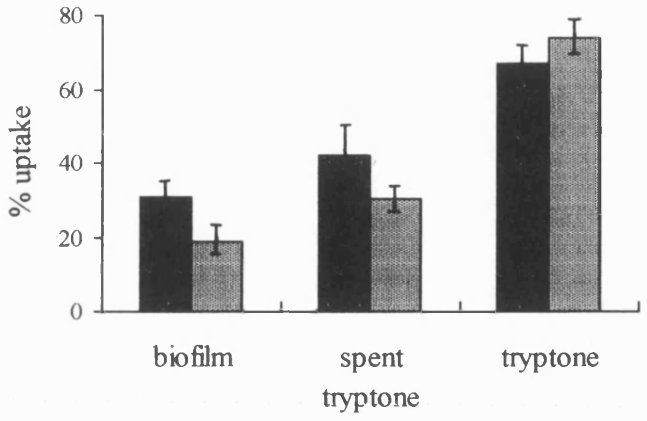

Figure 5.38 Chromium removal from 20 mg/l solution by F400 (■) and Picabiol (▒).

Seventy percent of the 20 mg/l Cr(VI) was accumulated by F400 in tryptone alone. Spent tryptone had a moderating influence, reducing the uptake to only 42%. The presence of biofilm organisms further reduced the uptake to only 32 % of the available Cr(VI). Plain tryptone had a lesser effect upon Picabiol than F400; 75 % of Cr was adsorbed from the 20 mg/l start concentration. The effect of spent tryptone and biofilm however, was more marked for Picabiol than F400, and the reduction in metal accumulation was substantial. Only 30 % and 20 % of Cr(VI) was removed from solution by Picabiol in the presence of spent tryptone and *K. pneumoniae.*

The degree of Cd(II) accumulation from a 5 mg/l solution by carbons in the presence of spent tryptone is depicted in Figure 5.39. Cadmium uptake was high, and almost 100% removal was achieved by Picabiol. The variation between biofilm covered, spent tryptone, and tryptone bathed Picabiol uptake was insignificant. A greater variation was noted with F400, where spent tryptone and *Klebsiella* biofilm enhanced Cd(II) uptake to 98 % from 93 % uptake after pre-adsorption of tryptone.

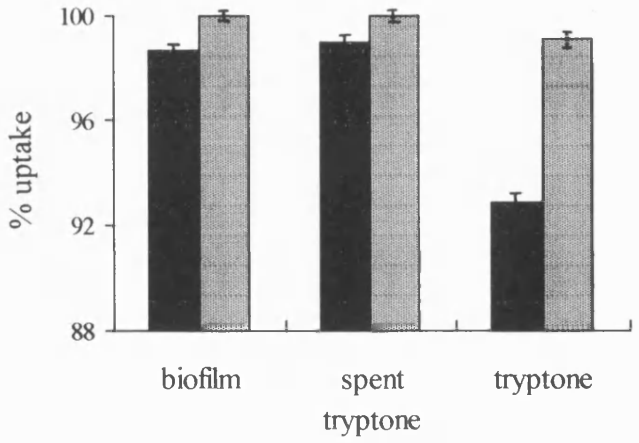

Figure 5.39 Cadmium removal from a 5 mg/l starting concentration by F400 (■) and Picabiol (▒).

In both cases the result of spent tryptone was to bring the mass adsorbed closer to that of the carbon with biofilm than that of tryptone medium alone. It is apparent that much of the adsorption attributed to the presence of the biofilm may indeed be due to changes in the culture medium which in turn alter the carbon surface characteristics (such as the charge and status of functional groups) brought about by metabolic activity of the cells, rather than true bioaccumulation or biosorption.

5.4.5 Equilibrium pH

The pH of the carbon uptake systems were difficult to control because of the interference with analysis, the metal chelation, and competitive adsorption to carbon which would result from use of buffers. The was some variation in the final pH of test solutions and so the pH of each flask was measured at equilibrium. The triplicate flasks for a given concentration of metal were in close agreement, and within 0.2 of a pH unit. Flasks were generally within the pH range of 5-7 at equilibrium. The presence of tryptone and biofilm produced a mild buffering; the pH of these flasks was between 6-7.

5.5 Summary data

A summary of pertinent results from Chapter 5 is given in Table 5.5.

Table 5.5 Summary of selected results.

Carbon	F400	Picabiol	Anthracite
Source material	bituminous coal	wood	bituminous coal
Activation status	steam activated	activated	non activated
Surface area (m^2/g)	118	50	17
Pore size distribution:			
Average pore d.	0.0257 μm	0.08779 μm	0.0452 μm
Pore d. ranges	60-2 μm 2.0-0.003 μm	25-8 μm 4.0-0.03 μm	0.02-0.003 μm
Porous nature	microporous	macroporous	little porosity
Biofilm development	fair on outer surface and in crevices only	good within larger pores of >5 μm	negligible
Metal loading			
Cr(VI)	high at high C_e only	high at high C_e only	negligible
Cd(II)	high loading even at low C_e	high loading even at low C_e	negligible
Ni(II)			negligible
Optimum conditions			
Cr(VI)	virgin carbon	virgin carbon	no influence
Cd(II)	biofilm coated	biofilm coated	no influence
Ni(II)	biofilm coated	biofilm coated	no influence

where d. = diameter
C_e = concentration at equilibrium

Discussion

6.1 Effects of experimental components

The components introduced to the batch equilibrium experiments for development of and maintenance of a *Klebsiella pneumoniae* subsp. *pneumoniae* biofilm were necessarily investigated for a) their effect upon metal analysis and b) their effect upon the metal uptake equilibrium achieved. The influence of components upon metal analysis detailed in Section 4.1.1 is discussed in Section 6.1.1, the effect of these components upon the metal uptake capacity of carbon is discussed in Section 6.4.1.

6.1.1 The effect upon metal analysis by AAS

The authors de Beer & Coetzee (1988) recommended atomic absorption spectrometry (AAS) for its specifity and low risk of interference when compared with other analysis techniques. The effect of components added throughout the experimental procedure or present on the carbon surface (culture media, carbon leachates and buffers) were unknown. These were investigated to discover whether the signal produced by AAS was enhanced or reduced in their presence. In this way the mass balance used to calculate the metal uptake was verified.

6.1.1.1 Culture media

The presence of culture medium caused falsely enhanced nickel signals by AAS. Nutrient broth (13 g/l) produced in excess of a 10 % enhancement in the Ni^{2+} determination, and tryptone (5 g/l) up to 10 % enhancement. Sterrit & Lester (1980) noted that many metals analysed by AAS could be subject to complexation and sequestration by incidental components of the sample, generally leading to erroneously low estimates of their concentration, but did not describe the enhancement of the Ni signal found in the current study. High concentrations of organic matter, Cl^- or S^{2-} in the metal matrix interfered with the AAS signal produced in the detection of Cd, Cu, Co and Pb. The effect was largely of signal suppression (Sterrit & Lester, 1980) reaching a maximum of 25 % signal suppression (Gallorini *et al.*, 1993). Gallorini and co-workers (1993) also provided evidence of signal *enhancement* specifically in the presence of S^{2-} (at a concentration of 0.1%) which caused a 10 % increase in the Cu signal.

The medium chosen for the cultivation of organisms in the biofilm batch equilibrium studies was a 5 g/l tryptone solution. This had a lesser effect on the signal produced by AAS due to the much lower concentration of NaCl in the medium (i.e. only 0.4 % w/w) when compared with other broths. Nutrient broth (NB) is composed of peptone, yeast and 'lab lemco' powder which have been implicated as causes of interference (Gadd & Griffiths, 1978). Each of the components of NB had higher intrinsic salt levels than tryptone, and an additional 5 g of NaCl in the 13 g of dried NB powder (i.e. 38 % w/w) used to produce 1 litre of the broth, raised the Na^+ and Cl^- ions to levels which could further interfere. At these heightened levels it was noted that the

flame conditions were significantly affected by the sodium ions: an orange flame was observed.

When assessed in the context of the experimental protocol, the effect of the low concentration of tryptone and leachate remaining in flasks, which may then have come into contact with the test metal solutions, was negligible. The accuracy of Ni, Cd, and Cr determinations by AAS in the range 5-30 mg/l was not compromised by any components introduced into the liquid phase by experimental practice, or leaching from either of the carbons, F400 or Picabiol, and so no re-calculation or normalization of data was required.

6.1.1.2 Buffers

Use of buffers *per se* was eliminated entirely not only for the interference effect upon metal measurement by AAS, since most buffers contain a metal component, but also because of their likely adsorption to active sites on the carbon and due to interactions with the biofilm organisms. Gadd & Griffiths (1978) highlighted the effects that may be induced by undefined components in a metal uptake.

The effect of the biological buffer, ¼ strength Ringer's solution, often used in biological systems to maintain cells in an isotonic environment was marked. The AAS determination of Cd was little affected by the presence of the buffer, but Ni measurement was enhanced by 7 % and sensitivity to Cr at high concentrations (i.e. 20 mg/l) was suppressed by as much as 20 %. This effect is likely due to the NaCl content of the buffer.

The pH buffers, such as phosphate and tris/HCl, were discarded by virtue of their metal content which caused interference in metal analysis and also competition for active sites on the carbon. The effect of buffer made it unacceptable for inclusion in the batch flasks particularly since unlike the growth medium its effect would not be diluted.

6.2 Bacterial resilience to metals

Gadd & Griffiths (1978) called into question research into metal interactions with micro-organisms, indicating that the isolation and maintenance of resistant strains by media containing peptones, yeast extracts and buffer solutions, could have significant effect upon the assessment of metal interactions.

6.2.1 Growth and metal uptake of planktonic cells

High concentrations of metal (i.e. 50-100 mg/l) suppressed the planktonic growth of the bacteria *K. pneumoniae*, *E. aerogenes* and the Gram-negative column isolate. Nickel was the metal least deleterious to survival of the organisms in this study yet only *E. aerogenes* would grow with concentrations of Ni at up to 100 mg/l. Metals present at sub-lethal levels, but generally in excess of 10 mg/l affect growth adversely by increasing the lag phase.

The simplest of growth media, tryptone, was used to assess the survival and proliferation of cells at the range of metal concentrations and in subsequent experiments. Components of more complex media such as citrate, cysteine, glutamate and EDTA are metal chelators and can have significant effect on microbial responses. These often allow cells to grow and proliferate in much higher metal concentrations than in their absence. In brewing systems the use of complex media has allowed cells to withstand much higher metal concentrations than minimal media and the presence of dead cells can have the same protective effect (Gadd & Griffiths, 1978; Remacle, 1990 a; Hughes & Poole, 1991).

The current study aimed to use a simple, if undefined, medium and keep conditions constant between the different organisms and metals such that the conditions of the metal challenge were comparable.

The choice of organisms for the equilibrium studies was made upon each organisms capacity for metal accumulation in addition to its survival ability. The unidentified Gram-negative rod which was able to survive well in the presence of metals, did not accumulate metals from solution. The Cr (VI) uptake of *Zoogloea ramigera* was

small but significant, yet cells did not accumulate Ni^{2+}. *Enterobacter aerogenes* although obviously able to withstand higher metal concentrations, and perhaps shock loads of up to 30 mg/l Cr (VI), 50 mg/l Ni (II) and 100 mg/l Cd (II), accumulated only Cd (II). This presents little potential for consideration in an application where a column might be challenged with a mix of metals.

The metal accumulation assay highlighted *K. pneumoniae* as the most efficient (of the 4 organisms tested) at removal of all three of the target metals, Cr (VI), Ni (II) and Cd (II). Scott & Palmer (1990) similarly found *Klebsiella* species to be superior to an *Arthrobacter* and *Pseudomonas* for Cd accumulation. This was probably due to external metal sequestration in the exopolysaccharides of *Klebsiella* rather than the intracellular uptake mediated by the other organisms. *Klebsiella pneumoniae* was used in the further studies where biofilm was developed over carbon.

The removal of metal ions from bulk solution was not taken as sufficient evidence of its uptake by cells. To prove that sorbed metal was accumulated by cells it was recovered by sonication, and re-introduced into bulk solution. Sonication is a harsh treatment and it is likely that all metals taken up by cells would have been released. Internalized metal would easily be liberated and pass through the filter as part of the cytoplasm, to be measured in bulk solution. Metal ions adsorbed by the components of the cell envelope, through a passive sorption may have been fully detached by sonication, or may remain bound to fragments of the disrupted envelope. These cell fragments could be small enough to remain in solution and pass through the filter to be detected in bulk solution. Sonication may have aided solubilization of the discrete exopolysaccharide capsule which acts as an ion exchange matrix enabling the metal to be recovered. In the standard accumulation experiments, diffuse exopolysaccharides with bound metal would remain remain associated with the organisms to be retained by filtration, and would not be found in the bulk solution.

6.2.2 Optimum culture age

Metal accumulation is not controlled purely by cell numbers but also the metabolic activity of the cells, their nutritional state and the phase of growth (among a concert of other effects) (Scott & Palmer, 1990). The greatest viable cell population of *K.*

pneumoniae was achieved after 48 hours, yet at this stage metal accumulation was at a minimum. The metal uptake capacity achieved a maximum coincident with the cell population reaching a minimum, after 9 days growth. This may be indicative of the older, starved cells of the population producing more of the metal accumulating exopolysaccharide matrix (Scott & Palmer, 1990).

The difference in metal uptake between younger and older cells may be indicative of the different modes of metal resistance; the younger cells perhaps relying upon metabolic activity and active transport to exclude metal ions, whereas older cells concentrate the toxic ions at the cell periphery. The passive accumulation does not require a metabolic activity and so is not expensive to maintain by cells in the stationary phase (Scott & Palmer, 1990; Fletcher, 1987; Gaylarde & Videla, 1992).

6.3 Biofilm studies

6.3.1 Electron microscopy preparation techniques

Of the three electron microscopy preparation techniques used, the method of cryo-fixation preserved the bacterial biofilm in the most life-like state (Jeffree & Read, 1991). This was borne out by the results where chemical fixation techniques removed all exopolysaccharide that was produced by the organisms colonizing the carbon surface. Freeze drying also introduced artifacts since the highly hydrated covering of exopolysaccharide necessarily becomes dehydrated by the process, leaving it with the appearance of strands linking the cells in an open network-like matrix. Authors in the past have suggested that this open network enables nutrients to be supplied deep into the biofilm, and is how access to the substratum can still be achieved even with confluent bacterial growth (Scott & Karanjkar, 1992). The advent of the cryo-fixation technique has dispelled these theories since this preparation involves very little dehydration; the cells are quickly frozen and any ice crystals sublimed off before gold coating. It is clear that the bacteria colonizing the carbon surface were swathed in a blanket of exopolysaccharide.

6.3.2 Carbon colonization

Comparison of two activated carbons (F400 and Picabiol) and a non-activated carbon (anthracite) demonstrated that activation changed the carbon characteristics in order to make it more favourable for colonization by *Klebsiella pneumoniae*. The main change through activation of carbon is the substantial increase in surface area, however the porosimetry studies demonstrated that much of the enhanced surface area of activated carbon was not accessible for microbial cells, also noted by Kida *et al.* (1990). In the case of F400 most of the pores were of diameter, 2.0-0.003 μm while microbial cells were up to 3 orders of magnitude larger than the smallest of these. Picabiol demonstrated a different pore size distribution, there being two distinct regions of porosity. The smaller pores (4.0 to 0.03 μm) were again, mainly unavailable for microbial colonization and so organisms were limited to the outer surface of the carbon and the larger pores (8-25 μm) which are remnants of the structure of the raw material; the cells of the woody precursor.

It is the nature of surface roughness through the size of the larger pores, leading to sites of shelter from shear forces that is the main factor involved in the different degree of biofilm development on the two carbons. The larger pores were visualized by scanning electron microscopy (SEM).

In a comparison of support media for development of anaerobic biofilms in acetate fed anaerobic reactors Fox *et al.* (1990) attributed the enhanced biofilm development on GAC (both in terms of rate and final mass and coverage by biofilm) to the nature of its surface roughness. The intrusions and crevices provided protection for the pioneer organisms and accumulated bacteria. Voice *et al.* (1992) also found enhanced biofilm on GAC rather than non-activated.

The enhancement of porosity, concomitant with an increase in surface area, does however play some role in the colonization. It was obvious that very little growth was present on the anthracite surface, (confirmed by the undetectable amounts of protein recovered from anthracite samples) a non-adsorbent carbon of low porosity.

Li & DiGiano (1983) noted that the start up times for biofilm growth on sand and GAC columns were different. The shorter time for biofilm development on GAC was attributed to adsorption of growth media deep in the pores of the carbon. This provided a nutrient rich micro-environment that was not acheivable on the non-adsorbent sand surface. Organisms are known to benefit from attachment through the supply of nutrients and protection, and obviously the activated carbon augments this (Olmstead & Weber, 1991).

Fox *et al.* (1990) suggested that a tendency of particles toward a spherical shape was advantageous. In the current study it may be true that the angular nature of anthracite particles increased the turbulence of the flow around them, increasing the shear force and inhibiting the biofilm development at the particle surface. Without crevices for initial attachment of pioneer organisms, subsequent colonization of the anthracite is likely to remain poor.

The author is in agreement with Fox *et al.* (1990) that the angular nature of anthracite particles are causative in poor biofilm development, however this is not the case for activated carbon substrates. The large, angular and irregular particles of Picabiol were more favourable for biofilm development than the smaller more spherical granules of F400. The protein assay data reinforced the qualitative assessment by SEM. Indeed it was the size and nature of the crevices, leading to the greater accessible surface area of the Picabiol, which is paramount in determining the degree of colonization and biofilm development.

Bacterial growth on carbon surfaces in the batch reactors was not confluent but consisted of small colonies or micro-colonies between surface features of the carbon, much in agreement of findings of Weber *et al.* (1978). Proliferation of biofilm would be expected in time, given favourable growth conditions. Other authors have achieved confluent growth under the rather different circumstances found in fluidized bed reactors (Voice *et al.*, 1992).

Kida *et al.* (1990) noted that it was roughness rather than surface area that determined biofilm development, but also suggested that surface charge was

important; generally negatively charged cells attach better to a surface with a positive charge.

Koch *et al.* (1991) noted a faster biofilm development upon activated carbon than sand particles but, unlike present study, found that eventually the biofilm on sand was more extensive than that on GAC. This may be due in some part to the fluidization of the beds or because a mixed culture was used. It would be difficult to discern which organism was favoured by the sand and GAC respectively, or indeed the growth characteristics of the unidentified organisms. A lack of suitable crevices in anthracite and sand, and hence their low surface area, was instrumental in the poor attachment of pioneer organisms and biofilm development.

6.4 Metal studies

6.4.1 Single metal isotherms

Activation is required for metal uptake. Metal accumulation by anthracite was negligible in comparison to the activated carbons F400 and Picabiol. The increase in surface area achieved by activation provides more functional groups which attract metal ions from solution. This is why the activated carbons Picabiol and F400, which have undergone the process to endow a micro-structure of pores and a greatly enhanced surface area, possess a greater capacity than the non-activated anthracite in all conditions.

The pH of solution is known to have a significant effect upon metal adsorption, however the equilibrium pH of the systems set up in the current study were grouped within a band from pH 5-7. This is generally accepted as the optimum pH region for metal sorption by the carbon (Seco *et al.*, 1997; Huang & Bowers, 1978). There is still some variation in Cr (VI) adsorption efficiency even with this narrow pH band although these variations in metal loadings were small when compared to the vast reduction in efficiency within the pH range 3-5. Alaerts *et al.*, (1989) noted that Cr (VI) removal from solution by a coconut based activated carbon was optimum between pH 2 and 7, but below pH 5 much of the removal was actually due to

reduction of Cr (VI) to Cr (III), a phenomenon also described by Kim & Zoltek (1977).

The change of uptake efficiency with pH was explained by Seco and co-workers (1997) in terms of the 'surface complex formation theory', where adsorption increased with pH because of the decrease in competition for active sites as the protons, and thus positive surface charge, decreased. This would result in a lower force of repulsion from the adsorbing surface for the metal ions and hence increased uptake.

The experimental system employed in the current study was necessarily simplified to ensure that the effect of each of the experimental components could be quantified. Additions to the batch flasks were limited to the growth medium, tryptone. This may be regarded as unrepresentative since in the natural environment metals would usually be complexed by chelating agents present in the soil or water which could include humic acid, fulvic acids and clay particles. Although the effects of such components were not investigated specifically, the presence of tryptone gave some indication of the consequences of a more complex menstrua.

The metal concentrations (between 5 and 100 mg/l starting concentration) were not sufficiently high for the activated carbons to become saturated. Should saturation have been achieved the slope of isotherms would have tended towards zero i.e they would plateau, as no further loading could be achieved even with increasing concentration. It was important that saturation point was not reached since the empirical Freundlich equation cannot accurately model this situation. In contrast the Langmuir model is based on the assumption of a monolayer coverage and saturation. At very high concentrations the Langmuir equation might be expected to better represent the experimental data.

6.4.1.1 Chromium

The loading of Cr (VI) on coconut based carbon screened by Alaerts and co-workers (1989) at a carbon dose of 1 g carbon/l and equilibrium concentration of 10 mg/l was 55 mg metal/g carbon. This was of the same order of magnitude as the lignite based

Picabiol, which at a carbon dose of 2.5 g/l and the same C_e, achieved a loading of 48 mg metal/g carbon, for virgin carbon without pre-adsorbed growth medium.

At the pH of the current batch equilibrium system, the chromium in solution should remain in the hexavalent state (and particularly $HCrO_4^-$) to be removed from solution mainly by adsorption (Huang & Bowers, 1978). The distribution of chromium species in solution at 10^{-5}M (comparable with the current experiments) measured by atom% is CrO_4^{2-} (24%), $HCrO_4^-$ (76%) at pH 6 and; CrO_4^{2-} (76%), $HCrO_4^-$ (24%) at pH 7, with no dichromate present (Shen-Yang & Ke-An, 1986). The current study bears out the findings of Corapcioglu & Huang (1987 b) which suggest that anionic binding would be significant at pHs below 7.

Factors affecting chromium (VI) availability

The presence of tryptone, spent tryptone, and biofilm, caused a reduction in the metal loading capacity of the carbon. Reduction of Cr (VI) to Cr (III) may lead to more chromium remaining in the bulk solution since Cr (III) does not adsorb well to carbon (Huang & Bowers, 1978). The pH of the tryptone system was generally slightly higher than that of water alone yet the reduction of Cr (VI) to Cr (III) in the presence of carbon is known to proceed at *lower* pH and particularly below pH 2.5 (Alaerts *et al.*, 1989). It is therefore unlikely that reduction of hexavalent chromium to Cr (III) plays a significant role at pH 5-7. It is more probable that some complexation and sequestration of anionic chromium has occurred. It is possible that there has some complexation of the Cr (VI) by components of the tryptone such as amino acids Although the concentration of tryptone in solution is low (having been rinsed from the reaction vessels before addition of the metal solution). No estimate of the likelihood and strength of such ligand interactions is possible, since no stability constants are available for the binding of organic ligands to Cr (VI), or the actual species CrO_4^{2-} and $HCrO_4^-$ present in solution.

Alternatively a swing in the relative concentration of the chromium species from CrO_4^{2-} to $HCrO_4^-$ with a small change in pH resulting from the presence of the tryptone and/or micro-organisms may have led to the adsorption of one of these

components at the expense of the other. Stability constants for these chromium species in support of this hypothesis are not yet available.

6.4.1.2 Divalent metals

The Cd (II) loadings from batch equilibrium studies were in accordance with those of other authors. The loading at an equilibrium concentration of 0.1 mg/l, calculated from the Freundlich constants supplied by Allen & Brown (1995) for lignite based activated carbon was 2.88 mg/g. The current study found loading at this concentration to be: F400; 0.258 mg/g, and Picabiol; 1.558 mg/g for virgin carbons, increasing in the presence of tryptone and biofilm.

Cadmium loading calculated from Langmiur constants described by Allen & Brown (1995) were at variance with estimates made by the Freundlich equation. This was probably due to the assumptions implicit in the Langmiur model, which is better suited to gas phase adsorption systems.

Cadmium speciation in solution is less complicated than that of chromium since the cadmium aqua ions are quite strong acids, and are hydrolysed in water. The predominant species at the concentrations and pHs of the experimental work is $CdOH^+$ (Cotton & Wilkinson, 1988). The main aqua ion of nickel is likely to be Ni^{2+} (aq) in the form of an octahedral complex i.e. $[Ni(OH_2)_6]^{2+}$ (Shriver *et al.*, 1994).

The cationic adsorption by carbon is known to increase abruptly at a specific pH and it is accepted that the optimum adsorption of Ni by F400 was achieved at pH > 8 (Corapcioglu & Huang, 1987 b). This may account for the poor adsorption of the metal cation at the pH range of the current study which is generally between pH 5 and pH 6. Cadmium adsorption also increases with increasing pH (Marzal *et al.*, 1996), and precipitation will occur at above pH 9 (LeyvaRamos *et al.*, 1997) which can lead to an erroneously high estimate of metal removal, although this will not be the case under currect experimental conditions.

Nickel loadings were slightly inferior to those of cadmium when assessed by mass, although the number of nickel ions were greater than the number of cadmium ions

adsorbed. Of the divalent metals, Ni was preferentially adsorbed from a ternary solution.

Factors affecting divalent metal availability

Unlike chromium adsorption by biofilm coated activated carbon, where the presence of a biofilm was detrimental to metal loading, biofilm had an enhanced effect upon the carbon sorption of divalent metals. The same phenomenon was experienced by Mohapatra and co-workers (1993) for uptake of another divalent metal, Cu^{2+}, by an active *Bacillus megaterium* biofilm. They described this phenomenon as synergy, although this was not the case since the biofilm and activated carbon together did not accumulate more than the sum of the component parts. The current study found no evidence of synergy, although biofilm did enhance the uptake of Cd and Ni by carbon.

It is known that a number of neutral ligands can displace some or all of the water molecules of the $[Ni(OH_2)_6]^{2+}$ ion to form complexes (Cotton & Wilkinson, 1988). The Ni^{2+} formation constants for a number of amino acids found in tryptone such as glycine and aspartic acid are high, indicating a high affinity of the metal ion for the ligand (Hughes & Poole, 1991), although the level of these amino acids in tryptone are low (Appendix 4) and sorption of Ni by biofilm and carbon was not affected adversely by tryptone residue.

The soft acid of Cd^{2+} would be subject to complexation by soft bases such as the sulphydryl groups of the amino acid cysteine (Hughes & Poole, 1991). The cysteine content of tryptone however, was very small, at only 0.22 w/w% of dry powder, which would leave only nanomolar quantities of cysteine in reaction vessels.

Thus it appears that the low level of the divalent metal uptake is more likely to be a function of the neutral pH rather than a decrease in the free metal ion concentration.

6.4.2 Molar basis isotherms

The compensation for molecular weight in the re-calculation of data had the effect of bringing metal capacities into narrower ranges than did the mass analysis also demonstrated to Allen & Brown (1995). The carbon capacities of Cd and Ni from the

single component isotherms, specifically in the presence of biofilm, coincided when replotted on a molar basis, i.e. capacities were the same, yet there was a distinct competition for their removal from a mixed metal solution.

Chromium uptake was greater than that of divalent metal in water systems. In the presence of tryptone and biofilm however, divalent metal uptake was superior to that of hexavalent chromium at concentrations generally above 50 μmol/l (tryptone) and 250-800 μmol/l for biofilm on F400 and Picabiol respectively.

6.4.3 Mixed metal isotherms

Chromium was preferentially adsorbed by carbon under the conditions of a ternary metal solution, with a concomitant reduction of divalent loading. Allen & Brown (1995) noted a similar effect; cadmium loading on a lignite carbon was substantially reduced when present in a multi-component system. Allen & Brown related the preferential adsorption of copper to the electronegativity of its ions. Copper possessed the greatest ionic potential and strongest attraction for the adsorbent. The complex surface chemistry of carbon rather precludes this simple explanation since the functional groups are heterogenous. Allen & Brown (1995) noted that Cu bound to carbon more readily than Cd and Zn putatively through its greater electrostatic attraction but no correlation was found with ionic radius or ionization energies.

The uptake of mixed metals with activated carbon presents added difficulties for interpretation. Comparison of the metal loadings on a molar basis provided a more accurate means of investigating competition in multi-component metal mixtures, since it is a measure of the total number of active sites available to each metal on the carbon.

In the F400 mixed metal/water system all metal loadings were suppressed by the presence of other metals. The loadings of all but the F400/Cd combination, were reduced below that of the single metals system for virgin carbons. In the presence of tryptone and biofilm, Cr loading upon both F400 and Picabiol was enhanced beyond its loading when present alone, whilst Ni and Cd loadings were suppressed.

6.4.4 Biofilm

Intuitively a mixture of metals might be thought more toxic to micro-organisms than single component waste at the same concentration. Yet the biological activity of heavy metals can be markedly affected by the presence of other ions. Cations such as magnesium and calcium can often reduce heavy metal inhibition, for example the toxic effects of nickel, cobalt, cadmium, zinc and manganese on *E. coli* were decreased in a medium with high Mg content. Competition from high levels of Mg reduced the uptake of the toxic metals (Gadd & Griffiths, 1978). The reduced metal loading from high concentration ternary metal solutions in the current work may indicate that the *K. pneumoniae* biofilm was indeed made more sensitive to the deleterious effects of the metal.

It is advantageous to maintain an active biofilm for a number of reasons which result in increased efficiency. The cell envelope, although in itself effective at accumulating metal, achieves only a fraction of the metal uptake of metabolizing cells (Gadd & Griffiths, 1978). In an industrial setting, using column operation, live cells within the biofilm ensure its constant regeneration should the biofilm be affected by toxic components from the waste stream. Biodegradable components of the waste stream can be used as an energy source in biomass production ensuring a further reduction in its toxicity, and there may be some limited regeneration of the carbon surface as pre-adsorbed components desorb and are utilized (Chudyk & Snoeyink, 1984).

6.4.5 Mechanisms of adsorption

The experiments indicate that adsorption by virgin carbon may be valence related. There was greater adsorption of hexavalent chromium than divalent cadmium and nickel ions.

At the pHs of the experimental work it is apparent that anion adsorption of chromium (VI) species such as CrO_4^{2-} is predominant on activated carbon. The aqua cations of Ni (II) and Cd (II) are poorly adsorbed when compared with the chromium capacity of the carbon. Molar uptakes of nickel and cadmium are similar, and it may be assumed from the water control experiments that the free metal in solution is the same for each metal since the pKa values are the same, i.e. 9.9. The affinity of the carbon

for the divalent metals is similar, and thus they would be expected to be subject to the same mechanism of adsorption.

6.5 Future work

The current study is a laboratory model using an organism, *Klebsiella pneumoniae*, which is a potential pathogen and would therefore not be used inan industrial setting for reasons of safety. Full elucidation of the extent and comparabilityof adsorption of chromium, nickel and cadmium by activated carbon and biofilm has not been possible due to the complex interactions of metals, the solution chemistry in the presence of activated carbon, micro-organisms and the ligands which may sequester metals. This concert of effects will alter both the bioavailability and the adsorption potential, and should be more fully characterized in future experiments.

6.5.1 Alternative sorbents

Many researchers are already involved in the screening of materials as potential metal sorbents. A range of waste materials such as bagasse (the waste from the sugar industry), sawdust and maize cob present themselves as cheap alternative adsorbents which would otherwise constitute disposal problems in themselves. The potential both as a support for bacterial biofilm growth and as a metal accumulator would need to be explored, as would the suitability of such systems for use with the current water treatment infrastructure which is the great advantage of activated carbon.

6.5.2 Buffered pH experiments

Clarification of the effect of pH could be made by buffered pH experiments, set at pHs from 3-9 at 0.5 unit intervals. Equilibrium pH should also be measured since it is expected that the buffering capacity of the chosen buffer, within the carbon system, would not be sufficient to maintain the set pH.

6.5.3 Co-adsorption of organics

A series of batch equilibrium experiments with metal and an organic component should be undertaken to find the full effect of metals and biofilm upon the carbon

uptake capacity for organic components. This requires careful use of controls to discover the relative importance of the carbon, the biofilm, and any changes in conditions.

The choice of the organic component should be made carefully. A pesticide, or industrial waste might be of interest, because of their persistence, mobility and toxicity in water courses and the wider environment. Organic components such as these may be found in the dumpsites, particularly in developing countries.

The interplay of adsorption, biosorption and bioregeneration of the biological carbon system would require a method of labelling the metal ions so as to chart the adsorption sites.

Use of a specific waste from a dumpsite could give an insight into how robust is the system, and how effective it would be in an environmental setting. This would require complete characterization of the waste.

6.5.4 Column studies

Batch studies in flasks can only give an estimate of how effective a certain mass of carbon would be for adsorption. Scale up into continuous column operation, using the parameters highlighted through batch studies is important to assess the full potential of a remediation system for field use. .

6.5.5 Disposal

Disposal of metal laden carbon remains a problem. A limited regeneration through heat fixing of metal laden biofilm onto the carbon surface followed by re-use was explored by Karanjkar & Scott (1993), but this involved no removal of the metals from the surface. Carbon suppliers regenerate saturated carbon by incineration and volatilization of the sorbed components. Carbon is rejected for regeneration when it reaches too high a metal content. This is because metals may desorb from the carbon leading to contamination of waters which may otherwise not have had a significant heavy metal content.

Metals are useful catalysts. Zeolites with a specific cage size and metal components are already used for catalyzing reactions and there may be some potential for re-use of metal laden carbon in the same way. There are precedents for carbon supported metal catalysts (Gandia & Montes, 1994; DomingoGarcia *et al.*, 1994; Rodriguez-Reinosa, 1995). It remains to discern whether metals laid down by adsorption or biosorption could be fixed suitably for re-use in this way.

Conclusions

- Screening of 4 Gram-negative bacteria chosen for their copious exopolysaccharide production highlighted one organism, *Klebsiella pneumoniae* subsp. *pneumoniae*, as having potential to withstand and proliferate in concetrations of Ni (II), Cd (II) and Cr(VI) at up to 50 mg/l. *Klebsiella pneumoniae* alone accumulated the 3 metals, so removing them from the bulk solution leading to a purification of the 'stream'.

- Biofilm development over carbon substrates was augmented by activation of the carbon and was due, in most part, to the enhanced surface area and its nature rather than an alteration of its chemistry. Colonization of the non activated carbon, anthracite, was poor and not detectable by protein assay.

- The nature of the carbon surface roughness is important in the development of a biofilm. Picabiol, although not in possession of as large a surface area as F400, was of a roughness and porosity suitable for enhanced biofilm attachment. Biofilm development on Picabiol was superior to that of F400 since it possessed crevices for attachment of pioneer organisms and a plethora of pores greater than 5 μm in diameter to provide shelter from shear forces. This enabled lateral proliferation of bacteria. Filtrasorb 400 in contrast had a huge surface area within pores too small to be accessible to the micro-organisms which were of length 1-2 μm.

- Activation was required for metal uptake. The uptake capacity of anthracite (non-activated carbon) was negligable. The combination of enhanced surface area and functional groups or active sites provided through activation endowed Picabiol and F400 with a comparatively high metal uptake capacity. The adsorption of Cd, Cr and Ni was described by the Freundlich isotherm.

- The mode and sites of adsorption may be different for the divalent and hexavalent metals. There may be different driving forces involved in chromium adsorption,

for it is known that below pH 7 there will at least be some reduction to Cr (III) which is less well adsorbed by carbon, although over the pH range of these experiments the active chromium species is likely to be $HCrO_4^-$.

- The isotherms of Cd and Ni were parallel, possibly demonstrating the same level of free metal in solution and a similar mode of adsorption to the carbon.

- The bacterial biofilm enhanced adsorption of Ni and Cd on Picabiol and F400 but reduced the adsorption of Cr on both carbons.

- A significant fraction of the metal removal of the biofilm systems was as a result of alterations in conditions due to the growth medium which was pre-adsorbed onto the carbon. This may have been due, in part, to a variation in pH or more probably a change of the surface functional groups or a chelating effect of components of the tryptone. A lack of published data regarding the stability constants of hexavalent chromium species with organic ligands made the assessment of free metal in solution difficult.

- Re-calculation of the mass basis isotherms on a molar basis resulted in metal capacities being compressed into narrower ranges so compensating for their differing molecular weights. This enabled a clearer view of competition in the system.

- The molar uptake of Cr from mixed metal solutions by Picabiol and F400 was reduced for water systems. On Picabiol the Ni and Cd capacities were also reduced in the presence of other metals, but on F400 in water the Ni loading was reduced whilst Cd loading was enhanced.

- For mixed metal tryptone and biofilm systems the molar Cr uptake was enhanced at the expense of Cd and Ni i.e. chromium was preferentially adsorbed on both Picabiol and F400.

- Changes in the composition of the tryptone through growth of organisms resulted in an alteration in the adsorption capacity of carbon possibly through a change in

the functional groups on the carbon surface. The effect of spent tryptone medium on metal uptake was intermediate to that of the biofilm and the tryptone control.

- At low Cr concentrations virgin Picabiol is most efficient, at very high Cr concentrations virgin F400 is an effective adsorbent. The high loadings of Cd and Ni on biofilm coated GAC maintained at low equilibrium concentrations, i.e. < 0.1 mg/l makes this efficient as a polisher of dilute waste streams.

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Appendices

Appendix 1

Growth media & buffer solutions

Nutrient broth (Code CM1, Oxoid Ltd., Basingstoke, U.K.)

'Lab Lemco' powder	1 g/l
Yeast extract powder	2 g/l
Peptone	5 g/l
Sodium chloride	5 g/l

pH 7.4 (approx)

Add 13 g to 1 litre of distilled water. Mix well and distribute into final containers. Sterilize by autoclaving at 121 °C for 15 min.

Ringer's solution (¼ strength) (Lab M, Amersham, Topley House, Bury, U.K.)

NaCl	2.25 g/l
KCl	0.105 g/l
$CaCl_2$	0.12 g/l
Na_2CO_3	0.05 g/l

Dissolve 1 tablet in 500 ml of distilled water.

Tris (hydroxymethyl) methylamine/HCl buffer 50 mM/l pH 7.2

A volume (44.7 ml) of 100 mM/l HCl (8.72 ml conc HCl diluted to 1 l) was added to 50 ml of 12.11 g/l tris (hydroxymethyl) methylamine and 5.3 ml distilled water.

Sodium phosphate buffer 200 mM/l pH 7.2.

A volume (28 ml) of NaH_2PO_4 (at 31.2 g/l) was added to 72 ml of 35.6 g/l $Na_2HPO_4.2H_20$ to give a 200 mM buffer at pH 7.2.

Typical analysis of peptone and hydrolysates (w/w)

	Tryptone	Peptone	Yeast Extract
Product code	L42	L37	L21
pH	7.3	7.2	7.0
Total nitrogen (%)	12.7	14	9.0
% NaCl	0.4	1.8	1.3
% K	0.4	3.6	7.0
Total Ca (ppm)	1127	690	351
Mg (ppm)	723	355	315
Fe (ppm)	36	88	62
Cu (ppm)	2	5	2
Pb (ppm)	0.6	0.4	0.7
Mn (ppm)	28	3.4	1.3
Sn (ppm)	1.7	1.0	3.0
Zn (ppm)	28	9.2	94
Total phosphorus (%)	1.5	0.8	2.7
Total lipids (%)	0.43	0.31	0.72

Appendix 2

Abbreviations

AAS	Atomic absorption spectrometry
ASTM	American Society for Testing and Materials
ATP	Adenine tri-phosphate
BATNEEC	Best available technique not entailing excessive cost
BSA	Bovine serum albumin
CDC	Chromium diphenyl carbazide
C_e	Equilibrium concentation (mg/l)
CFU	Colony forming units
COD	Chemical oxygen demand
d.H_2O	Distilled water
EDTA	Ethylenediaminetetraacetic acid
EEC	European Economic Community
EPS	Exopolysaccharide
F400	Filtrasorb 400, an activated carbon
g	Acceleration due to gravity
GAC	Granular activated carbon
ICP	Inductively coupled plasma
NB	Nutrient broth
NCIMB	National collection of industrial and marine bacteria
PAC	Powdered activated carbon
PACT	Powdered activated carbon treatment
rpm	Revolutions per minute
SD	Standard deviation
SDW	Sterile distilled water
SEM	Scanning electron microscopy
TOC	Total organic carbon
WHO	World Health Organization

Appendix 3

Units

Length	m	meter
	Å	Angstrom, 1×10^{-10} m
Mass	g	gramme
Amount	mol	mole
Volume	l	litre
Temperature	°C	Celcius
Pressure	psi	Pounds per square inch
	psia	Pounds per square inch absolute
Concentration	g/l	
	mol/l	
	ppm	parts per million
Specific surface area	m^2/g	
Intrusion	ml/g	
Loading	mg/g	

Prefixes

m	milli	$\times 10^{-3}$
μ	micro	$\times 10^{-6}$
n	nano	$\times 10^{-9}$
p	pico	$\times 10^{-12}$

Appendix 4

Amino acid analysis % (w/w)

Alanine	3.12
Arginine	5.53
Aspartic acid	7.31
Cysteine	0.22
Glutamic acid	17.61
Glycine	1.99
Isoleucine	2.51
Leucine	6.88
Lysine	7.17
Methionine	2.08
Phenylalanine	3.43
Proline	7.99
Serine	1.29
Threonine	1.87
Tryptophan	1.03
Tyrosine	3.10
Valine	5.47